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(54) Title: COMPOSITIONS, KITS, AND METHODS FOR EFFECTING ADENINE NUCLEOTIDE MODULATION OF DNA MISMATCH RECOGNITION PROTEINS

(57) Abstract

Compositions, and products comprising a MutS homolog which binds to a mismatched region of a duplex DNA molecule in the presence of ADP are provided, as are methods of binding MutS homologs to mismatched DNA in the presence of ADP. The use of MutL homolog derivatives in combination with MutS homologs is also included. Nonhuman mammals which are nullizygous for both *Msh2* and *p53* are also provided, as are methods of making and using the same.

COMPOSITIONS, KITS, AND METHODS FOR EFFECTING
ADENINE NUCLEOTIDE MODULATION OF
DNA MISMATCH RECOGNITION PROTEINS

FIELD OF THE INVENTION

5   The field of the invention is DNA mismatch protein binding, including

animals useful as models for tumorigenesis, apoptosis, and aging.

BACKGROUND OF THE INVENTION

DNA Mismatch Repair

The most widely accepted model for DNA post-replication mismatch

10   repair is based largely on the model of the DNA adenine methylation (Dam)-Instructed

pathway of *Escherichia coli* proposed by Modrich (1986, Basic Life Sci. 38:303-310;

Modrich, 1987, Ann. Rev. Biochem. 56:435-466; Modrich, 1989, J. Biol. Chem.

264:6597-6600; Modrich, 1991, Annu. Rev. Genet. 25:229-253; Modrich et al., 1996,

Annu. Rev. Biochem. 65:101-133). According to this model, the MutS protein

15   recognizes and binds mismatched nucleotides resulting from polymerase

misincorporation errors to form a MutS-DNA product (Su et al., 1986, Proc. Natl.

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Acad. Sci., USA 83:5057-5061; Su et al., 1988, J. Biol. Chem. 263:6829-6835). MutS

mismatch binding is followed by the interaction of MutL protein with the MutS-DNA

product (Grilley et al., 1990, Mutat. Res. 236:253-267), which accelerates ATP-

dependent translocation of the MutS-MutL complex (Allen et al., 1997, EMBO J.

5   16:4467-4476) to a hemimethylated GATC Dam site to which MutH protein is bound

(Welsh et al., 1987, J. Biol. Chem. 262:15624-15629; Au et al., 1992, J. Biol. Chem.

267:12142-12148). The MutS-MutL complex stimulates an intrinsic endonuclease

activity of MutH protein, which cleaves the non-methylated (i.e. more recently

replicated) DNA strand (Welsh et al., 1987, J. Biol. Chem. 262:15624-15629; Lahue et

10   al., 1987, Proc. Natl. Acad. Sci. USA 84:1482-1486; Su et al., 1989, Genome 31:104-

111; Cooper et al., 1993, J. Biol. Chem. 268:11823-11829; Grilley et al., 1993, J. Biol.

Chem. 268:11830-11837). Strand cleavage enables one of three single-stranded

exonucleases of *E. coli* (RecJ, ExoI, ExoVII) to degrade the non-methylated strand,

which can then be re-synthesized by the *E. coli* PolIII holoenzyme complex (Lahue et

15   al., 1989, Science 245:160-164). The net result is a strand-specific mismatch repair

event.

Many genetic studies performed using *E. coli* support this interpretation.

For example bacteria having a mutated *mutH*, *mutL*, or *mutS* gene exhibit a mutator

phenotype that is presumed to be the result of the increased probability of

20   misincorporation errors leading to mutations (Demerec et al., 1957, Bact. Genet.,

Carnegie Inst. Wash. Yearbook 370:390-406; Miyake, 1960, Genetics 45:755-762;

Siegel et al., 1967, J. Bacteriol. 94:38-47; Hill, 1970, Mutat. Res. 9:341-344).

However, not all predictions arising from the *E. coli* Dam-instructed model agree with

experimental results. For example, bacteria having a mutation in each of the *recJ*, *exoI*,

25   and *exoVII* genes do not exhibit a mutator phenotype, suggesting that other

exonuclease(s) or mechanism(s) are involved in the mismatch repair process.

Homologs of the procaryotic MutS and MutL proteins have been

identified in eukaryotes (Fishel et al., 1993, Cell 75:1027-1038; Prolla et al., 1994,

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Science 265:1091-1093; Bronner et al., 1994, Nature 368:258-261). MutH analogs appear to exist only in gram-negative bacteria.

Multiple MutS and MutL homologs have been identified in yeast and human cells which individually participate in such diverse activities as nuclear and

5   organelle mismatch repair as well as distinct meiotic functions (Fishel et al., 1997, Curr. Opin. Genet. Dev. 7:105-113). Germ-line mutations of the human MutS and MutL Homologs, hMSH2, hMLH1, and hPMS2, have been found to be associated with the common cancer predisposition syndrome, hereditary non-polyposis colorectal cancer (HNPCC; Bronner et al., 1994, Nature 368:258-261; Fishel et al., 1993, Cell

10   75:1027-1038). Yeast and human MutS and MutL homologs exist primarily as heterodimeric proteins. Yeast MSH2 protein has been found to be associated with MSH3 or MSH6, and yeast MLH1 has been found to be associated with PMS1. Human hMSH2 protein has been found to be associated with hMSH3 or hMSH6 (also designated GTBP or p160 by some authors), and human hMLH1 has been found to be

15   associated with hPMS2 (Li et al., 1995, Proc. Natl. Acad., Sci. USA 92: 1950-1954; Prolla et al., 1994, Science 265:1091-1093; Drummond et al., 1995, Science 268:1909-1912; Marsischky et al., 1996, Gen. Dev. 10:407-420; Acharya et al., 1996, Proc. Natl. Acad. Sci. USA 93:13629-13634). Furthermore, MSH2/MSH3 and MSH2/MSH6 protein complexes appear to possess overlapping and redundant mismatch binding

20   activities (Acharya et al., 1996, Proc. Natl. Acad. Sci. USA 93:13629-13634; Risinger et al., 1996, Nature Genet. 14:102-105).

Classification of MutS and MutL homologs is based on the presence in the proteins of highly conserved regions of amino acid identity. The most highly conserved region among MutS homologs includes approximately 150 amino acids

25   which comprise a helix-turn-helix domain associated with a Walker A adenine-nucleotide and magnesium binding motif (Walker et al., 1982, EMBO J. 1:945-951). This adenine nucleotide binding domain constitutes more than 80% of the identifiable homology between MutS homologs (Fishel et al., 1997, Curr. Opin. Genet. Dev. 7:105-113). Both purified bacterial MutS homologs and purified yeast MutS homologs

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possess an intrinsic low-level ATPase activity (Haber et al., 1991, EMBO. J. 10:2707-2715; Chi et al., 1994, J. Biol. Chem. 269: 29993-29997; Chi et al., 1994, J. Biol. Chem. 269:29984-29992; Alani et al., 1997, Mol. Cell Biol. 1 7: 2436-2447). This ATPase activity is likely to be important for the function of MutS homologs, as

5   indicated by the fact that mutation of conserved amino acid residues in the adenine nucleotide binding domain results in a dominant mutator phenotype in both bacteria and yeast (Haber et al., 1991, EMBO. J. 10:2707-2715; Wu et al., 1994, J. Bacteriol 176:5393-5400; Alani et al., 1997, Mol. Cell Biol. 1 7: 2436-2447). A central role for the adenine nucleotide binding domain is consistent with the ATP-dependent

10   translocation model of mismatch repair proposed by Modrich and colleagues (Allen et al., 1997, EMBO J. 16:4467-4476).

Genetic and biochemical studies of the human mismatch repair process indicate that it is similar to bacterial mismatch repair, except that the physiologically relevant mechanism for directing strand specificity is unknown (Miller et al., 1976,

15   Proc. Natl. Acad. Sci. USA 73:3073-3077; Glazer et al., 1987, Mol. Cell. Biol., 7:218-224; Holmes et al., 1990, Proc. Natl. Acad. Sci. USA 87:5837-5841; Thomas et al., 1991, J. Biol. Chem. 266:3744-3751; Fang et al., 1993, J. Biol. Chem. 268:11838-11844; Longley et al., 1997, J. Biol. Chem. 272: 10917-10921). Purified hMSH2 protein binds mismatched nucleotides and DNA lesions (Fishel et al., 1994, Science

20   266:1403-1405; Fishel et al., 1994, Cancer Res. 54:5539-5542; Mello et al., 1996, Chemistry & Biology 3:579-589), and the specificity and affinity of that recognition is enhanced by association of hMSH2 with hMSH3 or hMSH6 (Drummond et al., 1995; Acharya et al., 1996, Proc. Natl. Acad. Sci. USA 93:13629-13634; Palombo et al., 1996, Curr. Biol. 6:1181-1184).

25   Although the ability of MutS homologs to bind to mismatched duplex DNA has been recognized (e.g. U.S. Patent No. 5,556,750), methods of using MutS homologs *in vitro* have been limited by a lack of understanding regarding the properties of such homologs. A need remains for methods of binding MutS homologs and

mismatched duplex DNA, which methods take advantages of the biochemical

properties of such homologs.

Transgenic and Nullizygous Animals

The development of transgenic animals and nullizygous animal models

5   has provided important new avenues for the study of specific gene functions in

differentiation, embryogenesis and neoplastic development (Palmiter et al., 1986, Ann.

Rev. Genet. 20:465-499). Transgenic animals frequently serve as model systems for

the study of various disease states and also provide an experimental system in which to

test compounds for their ability to regulate disease. Nullizygous animals are similarly

10   useful as experimental systems for the testing of compounds useful for diagnosis,

treatment, or both, of disease.

Lukkarinen et al. (1997, Stroke 28:639-645) teaches that gene constructs

which enable the generation of transgenic mice also enable the generation of other

transgenic rodents, including rats. Similarly, nullizygous mutations in a genetic locus

15   of an animal of one species can be replicated in an animal of another species having a

genetic locus highly homologous to the first species. For example, many genetic loci

are highly homologous among mammals, and even more highly homologous among

subgroups of mammals, such as among rodents.

The mutator hypothesis of tumorigenesis suggests that loss in an

20   organism of a chromosomal stability function, a chromosomal maintenance function, or

both, results in an elevated mutation rate in the organism. An elevated mutation rate

hastens accumulation of the numerous mutations required for multistep carcinogenesis

(Loeb, 1991, Cancer Res. 51:3075-3079).

Loss of the function of p53 protein has been proposed to increase

25   cellular hypermutability in an organism, thereby accelerating tumorigenesis, although a

clear role for p53 protein in genomic instability remains controversial (Kastan et al.,

1992, Cell 71:587-597; Fishel et al., 1997, Curr. Opin. Genet. Dev. 7:105-113). *p53*,

the gene encoding p53 protein, is frequently mutated in a wide range of human cancers

including, but not limited to, colonic tumors (Fearon et al., 1990, Cell 61:759-767).

Transgenic mice nullizygous for *p53* are viable and susceptible to tumorigenesis (de

Wind et al., 1995, Cell 82:321-330; Reitmair et al., 1995, Nature Genet. 11:64-70;

Donehower et al., 1992, Nature 356:215-221; Jacks et al., 1994, Curr. Biol. 4:1-7;

Purdie et al., 1994, Oncogene 9:603-609).

5   Although nullizygous *p53* mice can be used as models of

carcinogenesis, the rates at which such mice develop tumors can be slower than what is

desirable, particularly for large-scale screening studies involving numerous potential

anti-cancer therapeutic or prophylactic compositions. What is needed is a transgenic

mouse which, when exposed to a carcinogen, succumbs to tumorigenesis caused by the

10   carcinogen more readily than does a nullizygous *p53* mouse and which, even when not

exposed to an identifiable carcinogen, succumbs to tumors more readily than does a

nullizygous *p53* mouse.

               Critical unmet needs also exist for animal models of programmed cell

death (apoptosis) and of aging.

15   The present invention satisfies the needs identified above.

                          SUMMARY OF THE INVENTION

               The invention relates to a method of modifying a mismatched duplex

DNA. The method comprises contacting an MSH dimer and the mismatched duplex

DNA in the presence of a binding solution. In one embodiment, the binding solution

20   comprising a nucleotide selected from the group consisting of ADP and ATP, and the

concentration of ATP in the binding solution is less than about 3 micromolar The

MSH dimer thereby associates with the mismatched region of the mismatched duplex

DNA, and the mismatched duplex DNA is modified. In one embodiment, the MSH

dimer is selected from the group consisting of a prokaryotic MSH homodimer, a

25   prokaryotic MSH heterodimer, a eukaryotic MSH homodimer, and a eukaryotic MSH

heterodimer. The MSH dimer may, for example, be a homodimer of a MutS homolog

selected from the group consisting of a human MutS homolog, a murine MutS

homolog, a rat MutS homolog, a *Drosophila* MutS homolog, a yeast MutS homolog,

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and a *Saccharomyces cerevisiae* MutS homolog. An example of a eukaryotic MSH homodimer is an MSH2 homodimer. The eukaryotic MSH heterodimer useful in this method comprises MutS homologs independently selected from the group consisting of an MSH2 protein, an MSH3 protein, an MSH4 protein, an MSH5 protein, and an

5   MSH6 protein. By way of example, the MSH dimer may be selected from the group consisting of an MSH2:MSH3 heterodimer, an MSH2:MSH6 heterodimer, and an MSH4:MSH5 heterodimer. In another embodiment of this method, the prokaryotic MSH dimer is a homodimer of *Escherichia coli* MutS. Preferably, the MSH dimer is substantially purified.

10   According to this method, the concentration of ATP in the binding solution is preferably less than about 0.3 micromolar, or, more preferably, the binding solution is substantially free of ATP. In another embodiment of this method, at least one of the MSH dimer and the mismatched duplex DNA is bound to a support. In yet another embodiment, the mismatched duplex DNA has at least one free end. In still

15   another embodiment, the mismatched duplex DNA comprises a DNA strand generated by reverse transcription of mRNA obtained from an organism.

         According to one aspect of this method, the mismatched duplex DNA comprises a first DNA strand having a reference nucleotide sequence and a second DNA strand. The second strand may, for example, be selected from the group

20   consisting of a DNA strand obtained from an organism, a DNA strand obtained by amplification of at least a portion of a polynucleotide obtained from an organism, a DNA strand obtained by cleavage of a polynucleotide obtained from an organism, and a DNA strand obtained by reverse transcription of a polynucleotide obtained from an organism. The second DNA strand may also comprise at least a portion of a gene

25   associated with a cancer in the organism. In one embodiment, the organism is a human and the gene is selected from the group consisting of an oncogene and a tumor suppressor gene. By way of example, such genes include *abl, akt2, apc, bcl2α, bcl2β, bcl3, bcr, brca1, brca2, cbl, ccnd1, cdk4, crk-II, csf1r/fms, dbl, dcc, dpc4/smad4, e-cad, e2f1/rbap, egfr/erbb-1, elk1, elk3, eph, erg, ets1, ets2, fer, fgr/src2, fli1/ergb2, fos,*

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fps/fes, fra1, fra2, fyn, hck, hek, her2/erbb-2/neu, her3/erbb-3, her4/erbb-4, hras1, hst2, hstf1, ink4a, ink4b, int2/fgf3, jun, junb, jund, kip2, kit, kras2a, kras2b, lck, lyn, mas, max, mcc, met, mlh1, mos, msh2, msh3, msh6, myb, myba, mybb, myc, mycl1, mycn, nf1, nf2, nras, p53, pdgfb, pim1, pms1, pms2, ptc, pten, raf1, rb1, rel, ret, ros1, ski, src1, tal1, tgfbr2, thra1, thrb, tiam1, trk, vav, vhl, waf1, wnt1, wnt2, wt1, and *yes1.*
Preferably, the cancer is hereditary non-polyposis colon cancer and the gene is selected from the group consisting of *mlh1, msh2, msh3, msh6, pms1,* and *pms2.* Alternately, the cancer may be selected from the group consisting of a leukemia, a lymphoma, a meningioma, a mixed tumor of a salivary gland, an adenoma, a carcinoma, an adenocarcinoma, a sarcoma, a dysgerminoma, a retinoblastoma, a Wilms' tumor, a neuroblastoma, a melanoma, and a mesothelioma.

In another aspect of this method, the mismatched duplex DNA and the MSH dimer are contacted in the presence of at least one non-mismatched duplex DNA. According to this aspect, the method may further comprise separating the MSH dimer from the non-mismatched duplex DNA after contacting the mismatched duplex DNA and the MSH dimer. In one embodiment, the method further comprising dissociating the mismatched duplex DNA and the MSH dimer after separating the MSH dimer from the non-mismatched duplex DNA and thereafter amplifying the mismatched duplex DNA. The MSH dimer may be bound to a support prior to separating the non-mismatched duplex DNA from the MSH dimer and the non-mismatched duplex DNA is separated from the MSH dimer in the presence of a separating solution which is substantially free of ATP. In one embodiment, this method further comprises releasing the mismatched duplex DNA from the MSH dimer after separating the non-mismatched duplex DNA from the MSH dimer. If the mismatched duplex DNA has at least one free end, it may be released from the MSH dimer by contacting the MSH dimer with a releasing solution. The releasing solution may, for example, be selected from the group consisting of a solution comprising ATP and Mg^{2+} ions, a solution comprising ATP and a magnesium-chelating agent, a solution comprising high salt, a solution comprising a gamma-modified ATP analog and Mg^{2+} ions, and a solution

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comprising a gamma-hydrolysis-resistant ATP analog and Mg^{2+} ions. Preferably, the releasing solution comprises ATP and Mg^{2+} ions. If the mismatched duplex DNA does not have a free end, it may be released from the MSH dimer by contacting the MSH dimer with a releasing solution. This releasing solution may be selected from the

5   group consisting of a solution comprising a magnesium-chelating agent, a solution comprising high salt, a solution comprising a double-stranded DNA cleaving enzyme, ATP and Mg^{2+} ions, a solution comprising a double-stranded DNA cleaving enzyme, a gamma-modified ATP analog, and Mg^{2+} ions, and a solution comprising a double-stranded DNA cleaving enzyme, a gamma-hydrolysis-resistant ATP analog, and Mg^{2+}

10   ions. According to one embodiment, after contacting the mismatched DNA and the MSH dimer, the MSH dimer may be contacted with a MutL homolog.

In another aspect of this method, association of the MSH dimer with the mismatched duplex DNA is detected after or while contacting the MSH dimer with the mismatched duplex DNA. Association of the MSH dimer with the mismatched duplex

15   DNA may be detected, for example, using an assay selected from the group consisting of a gel mobility shift assay, a filter binding assay, an immunological assay, a sedimentation centrifugation assay, a spectroscopic assay, an optical affinity assay, a DNA footprint assay, and a nucleolytic cleavage protection assay.

In still another aspect of this method, the duplex DNA with which the

20   MSH dimer is contacted does not have a free end. If the MSH dimer is present in molar excess with respect to the mismatched duplex DNA, then an average of more than one the MSH dimer associates with one molecule of the mismatched duplex DNA.

The invention also includes a method of modifying a mismatched duplex DNA which does not have a free end. This method comprising contacting the

25   mismatched duplex DNA and an MSH dimer having ADP bound thereto in the presence of a binding solution. The concentration of ATP in the binding solution is less than about 3 micromolar, and the homolog associates with the mismatched region of the mismatched duplex DNA, thereby modifying the mismatched duplex DNA.

The invention further includes a method of segregating a mismatched duplex DNA from a population of DNA molecules. The method comprises contacting an MSH dimer and the population in the presence of a binding solution and segregating the MSH dimer from the population. The binding solution comprises a nucleotide

5   selected from the group consisting of ADP and ATP, an the concentration of ATP in the binding solution is less than about 3 micromolar. The MSH dimer associates with the duplex DNA in the presence of the binding solution. When the MSH dimer is segregated from the population, the mismatched duplex DNA is also segregated from the population.

10   The invention still further includes a method of detecting a difference between a sample nucleotide sequence and a reference nucleotide sequence. According to this method, a first DNA strand and a second DNA strand are annealed to form a duplex DNA. The first DNA strand has the sample nucleotide sequence, and the second DNA strand has a nucleotide sequence which is complementary to the reference

15   nucleotide sequence. If there is a difference between the sample nucleotide sequence and the reference nucleotide sequence, then the duplex DNA is a mismatched duplex DNA. The duplex DNA and an MSH dimer are contacted in the presence of a binding solution comprising a nucleotide selected from the group consisting of ADP and ATP. The concentration of ATP in the binding solution is less than about 3 micromolar, and

20   the MSH dimer associates with the duplex DNA if the duplex DNA is a mismatched duplex DNA. According to this method, it is then determined whether the MSH dimer is associated with the duplex DNA molecule. Association of the MSH dimer with the duplex DNA molecule is an indication that there is a difference between the sample nucleotide sequence and the reference nucleotide sequence.

25   In addition, the invention includes a kit for separating a mismatched duplex DNA from non-mismatched duplex DNAs. The kit comprises at least two MutS homologs, a linker for binding the at least one of the MutS homologs to a support, and an additional reagent. The reagent may, for example, be selected from the group consisting of a nucleotide and a releasing solution, wherein the nucleotide is

selected from the group consisting of ADP and ATP, and wherein the releasing

solution comprises Mg^{2+} and a compound selected from the group consisting of ATP,

a gamma-modified ATP analog, and a gamma-hydrolysis-resistant ATP analog.

The invention also includes a method of determining whether a mammal

5   is predisposed for carcinogenesis. This method comprises annealing a first DNA strand

and a second DNA strand to form a duplex DNA. The first DNA strand has the

nucleotide sequence of at least a portion of a gene selected from the group consisting of

an oncogene and a tumor suppressor gene of the mammal. The second DNA strand has

a nucleotide sequence which is complementary to the consensus nucleotide sequence of

10   this region. If there is a sequence difference between the first DNA strand and the

second DNA strand then the duplex DNA is a mismatched duplex DNA. The duplex

DNA and an MSH dimer are contacted in the presence of a binding solution

comprising a nucleotide selected from the group consisting of ADP and ATP. The

concentration of ATP in the binding solution is less than about 3 micromolar, and the

15   MSH dimer associates with the duplex DNA if the duplex DNA is a mismatched

duplex DNA. According to this method, it is determined whether the MSH dimer is

associated with the duplex DNA, whereby association of the MSH dimer with the

duplex DNA is an indication that the mammal is predisposed for carcinogenesis.

The invention further includes a method of fractionating a population of

20   duplex DNAs. This method comprises contacting the population with an MSH dimer

in the presence of a binding solution comprising a nucleotide selected from the group

consisting of ADP and ATP. The concentration of ATP in the binding solution is less

than about 3 micromolar, and the MSH dimer associates with at least one mismatched

duplex DNA in the population. The MSH dimer is segregated from the population of

25   duplex DNAs, whereby the mismatched duplex DNA is also segregated from the

population. The population is thereby fractionated.

The invention still further includes a method of selectively amplifying at

least one mismatched duplex DNA of a population of duplex DNAs. This method

comprises contacting the population with an MSH dimer in the presence of a binding

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solution comprising a nucleotide selected from the group consisting of ADP and ATP. The concentration of ATP in the binding solution is less than about 3 micromolar, and the MSH dimer associates with the mismatched duplex DNA. The MSH dimer is thereafter segregated from the population of duplex DNAs, whereby the mismatched

5   duplex DNA is also segregated from the population of duplex DNAs. The mismatched duplex DNA is then amplified, whereby the mismatched duplex DNA is selectively amplified.

The invention also includes a method of determining whether the nucleotide sequence of a first copy of a genomic sequence differs from the nucleotide

10   sequence of a second copy of the genomic sequence. This method comprises amplifying a region of each of the first copy and the second copy of the genomic sequence to yield amplified first copies and amplified second copies. The amplified first copies and the amplified second copies are mixed and denatured to form a first mixture. The nucleic acids in the first mixture are then annealed to form a second

15   mixture comprising duplex DNAs. If the nucleotide sequence of first copy and the nucleotide sequence of the second copy of the genomic sequence differ, then at least some of the duplex DNAs in the second mixture are mismatched duplex DNAs. The annealed second mixture is contacted with an MSH dimer in the presence of a binding solution comprising a nucleotide selected from the group consisting of ADP and ATP.

20   The concentration of ATP in the binding solution is preferably less than about 3 micromolar, whereby the MSH dimer associates with mismatched duplex DNA. According to this method, it is then determined whether the MSH dimer is associated with at least some of the duplex DNAs. Association of the MSH dimer with at least some of the duplex DNAs is an indication that the nucleotide sequence of the first copy

25   of the genomic sequence differs from the nucleotide sequence of the second copy of the genomic sequence.

The invention further includes a composition for segregating a mismatched duplex DNA from a population of duplex DNAs. The composition comprises an MSH heterodimer bound to a support.

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The invention still further includes a kit for screening a genomic region for a nucleotide sequence which differs from a reference nucleotide sequence. This kit comprises a pair of primers complementary to the ends of the region for amplifying the region, a DNA strand having the reference nucleotide sequence, and at least two MutS

5   homologs.

The invention yet further relates to a nonhuman mammal which is nullizygous for both *Msh2* and *p53*. The mammal does not express *Msh2* or *p53* and exhibits a phenotype selected from the group consisting of inappropriate fetal apoptosis and a predisposition for carcinogenesis.

10   The invention also relates to a method of making a nonhuman mammal which is nullizygous for both *Msh2* and *p53*, does not express *Msh2* or *p53*, and exhibits a phenotype selected from the group consisting of a predisposition for inappropriate fetal apoptosis and a predisposition for carcinogenesis. This method comprises mating

15   a) a first parent mammal which comprises at least one null allele of *Msh2* and at least one null allele of *p53* and

b) a second parent mammal comprising at least one null allele of *Msh2* and at least one null allele of *p53*. As a result of this mating, a non-human mammal is generated which is nullizygous for both *Msh2* and *p53*, does not express *Msh2* or *p53*,

20   and exhibits a phenotype selected from the group consisting of inappropriate fetal apoptosis and a predisposition for carcinogenesis.

The invention further relates to a method of determining whether a compound affects tumorigenesis in mammals. This method comprises administering the compound to a first nonhuman mammal which is nullizygous for both *Msh2* and

25   *p53*, does not express *Msh2* or *p53*, and exhibits a predisposition for carcinogenesis. Tumor incidence in the first nonhuman mammal is compared with tumor incidence in a second nonhuman mammal of the same type which is nullizygous for both *Msh2* and *p53*, does not express *Msh2* or *p53*, exhibits a predisposition for carcinogenesis, and to which the compound is not administered. A difference in tumor incidence in the first

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transgenic mammal compared with tumor incidence in the second transgenic mammal
is an indication that the compound affects tumorigenesis in mammals.

The invention still further relates to a method of determining whether a
compound affects a biological phenomenon in mammals. The phenomenon may, for
example, be selected from the group consisting of apoptosis, aging, and fetal
development. The method comprises administering the compound *in utero* to a first
nonhuman mammalian embryo which is nullizygous for both *Msh2* and *p53*, does not
express *Msh2* or *p53*, and exhibits a predisposition for inappropriate fetal apoptosis.
The development of the first nonhuman mammalian embryo is compared with the
development of a second nonhuman mammalian embryo of the same type which is
nullizygous for both *Msh2* and *p53*, does not express *Msh2* or *p53*, exhibits a
predisposition for inappropriate fetal apoptosis; and to which the compound is not
administered. A difference in the development of the first nonhuman mammalian
embryo compared with the development of the second nonhuman mammalian embryo
is an indication that the compound affects the biological phenomenon in mammals.

The invention yet further relates to a cell line which is nullizygous for
both *Msh2* and *p53*, does not express *Msh2* or *p53*, and exhibits a phenotype selected
from the group consisting of a predisposition for carcinogenesis and a predisposition
for apoptosis. The cell line is made by culturing a cell obtained from the nonhuman
mammal described herein.

The invention also relates to a method of determining whether a
composition affects expression of a gene selected from the group consisting of the *p53*
gene and a gene encoding a MutS homolog. This method comprising administering the
composition to a first non-human mammal which is nullizygous for one of the *p53*
gene and the gene encoding a MutS homolog. A phenotype of the non-human mammal
is compared with the phenotype of a second non-human mammal of the same type
which is not nullizygous for the one of the *p53* gene and the gene encoding a MutS
homolog, wherein the phenotype is selected from the group consisting of inappropriate
fetal apoptosis and a predisposition for carcinogenesis. A difference between the

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phenotype of the first non-human mammal and the phenotype of the second non-human mammal is an indication that the composition affects expression of the other of the *p53* gene and the gene encoding a MutS homolog.

The invention further relates to a method of determining whether a

5   composition affects expression of a gene selected from the group consisting of the *p53* gene and a gene encoding a MutS homolog. This method comprises administering the composition to a first cell derived from a non-human mammal which is nullizygous for one of the *p53* gene and the gene encoding a MutS homolog. A phenotype of the first cell is compared with the phenotype of a second cell derived from a non-human

10   mammal of the same type which is not nullizygous for the one of the *p53* gene and the gene encoding a MutS homolog, wherein the phenotype is selected from the group consisting of inappropriate fetal apoptosis and a predisposition for carcinogenesis. A difference between the phenotype of the first cell and the phenotype of the second cell is an indication that the composition affects expression of the other of the *p53* gene and

15   the gene encoding a MutS homolog.

The invention still further relates to a composition comprising a human MutS homolog fragment, wherein the fragment comprises a MutS homolog interaction region.

The invention yet further relates to a method of inhibiting association of

20   a first human MutS homolog and a second human MutS homolog. This method comprises contacting at least one of the first human MutS homolog and the second human MutS homolog with a human MutS homolog fragment comprising a MutS homolog interaction region. Inhibition of the first and the second human MutS homologs is thus inhibited.

25   The invention also relates to a composition comprising substantially purified hMSH5.

The invention further relates to a composition comprising an isolated nucleic acid encoding hMSH5.

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The invention still further includes an alternate method of modifying a mismatched duplex DNA. This method comprises contacting an MSH dimer and the mismatched duplex DNA in the presence of a binding solution comprising ADP. The concentration of ADP in the binding solution is at least about ten times the

5   concentration of ATP, if ATP is present in the binding solution. The MSH dimer thereby associates with the mismatched region of the mismatched duplex DNA and modifies the mismatched duplex DNA.

BRIEF DESCRIPTION OF THE DRAWINGS

Figure 1, comprising Figures 1A, 1B, 1C, 1D, 1E, and 1F, depict

10   binding of hMSH2:hMSH6 heterodimer to mismatched and non-mismatched duplex DNA. Figure 1A is an image of the results of a gel mobility shift assay performed using the G/T-mismatched 81-base pair duplex DNA substrate described herein. The concentrations of heterodimer used in the assay are indicated along the top of the image. The position of the S-shifted electrophoretic band is indicated by "S". Figure

15   1E is a graph which depicts the relationship between the concentration of heterodimer and the amount of product corresponding to the S-shifted electrophoretic band in Figure 1A, as assessed using a phosphoimaging device. Figure 1B is an image of the results of a gel mobility shift assay performed using the homologous 81-base pair duplex DNA substrate described herein. The concentrations of heterodimer used in the

20   assay are indicated along the top of the image. The position of the NS-shifted electrophoretic band is indicated by "NS". Figure 1F is a graph which depicts the relationship between the concentration of heterodimer and the amount of product corresponding to the NS-shifted electrophoretic band in Figure 1B, as assessed using a phosphoimaging device. Figure 1C is an image which depicts the results of a DNase

25   footprint assay performed using the 81-base pair G/T-mismatched duplex DNA substrate described herein. The concentrations of 81-base pair are indicated along the top of the image. The position of the G residue of the G/T-mismatched substrate is indicated by "G", and the approximate region of the substrate protected from DNase

cleavage by the heterodimer is indicated by a vertical line. Figure 1D is an image
which depicts the results of a DNase footprint assay performed using the homologous
81-base pair duplex DNA substrate described herein. The concentrations of
heterodimer used in the assay are indicated along the top of the image. The position of
5   the G/C base pair corresponding to the G/T-mismatched base pair of the mismatched
substrate is indicated by "G".

Figure 2, comprising Figures 2A, 2B, 2C, and 2D, depicts the results of
gel mobility shift assays used to assess the ability of various adenine nucleotides to
dissociate MSH dimer from the mismatch site, corresponding to the S-shifted
10   electrophoretic band, such that the MSH dimer, corresponding to the NS-shifted
electrophoretic band, exhibited DNA-associated diffusion. Figure 2A is an image of an
assay in which the product corresponding to the S-shifted electrophoretic band was
incubated in the presence of ATP at the concentration listed along the top of the image.
Figure 2B is an image of an assay in which the product corresponding to the S-shifted
15   electrophoretic band was incubated in the presence of adenosine-5'-O-3'-
thiotriphosphate (ATP-γ-S) at the concentration listed along the top of the image.
Figure 2C is an image of an assay in which the product corresponding to the S-shifted
electrophoretic band was incubated in the presence of ADP at the concentration listed
along the top of the image. In Figures 2A, 2B, and 2C, "-" indicates that no
20   heterodimer was included in the assay mixture. Figure 2D is a graph which depicts
quantitated results obtained using the results depicted in Figures 2A, 2B, and 2C, as
assessed using a phosphoimaging device.

Figure 3 is a bar graph which depicts the effect of selected nucleotides,
deoxynucleotides, and nucleotide analogs on G/T mismatch binding by the
25   heterodimer, relative to the degree of binding observed in the absence of a
(deoxy)nucleotide or analog. The effect of each indicated (deoxy)nucleotide or analog
was assessed at 25 micromolar (left bar of each pair) and at 250 micromolar (right bar
of each pair).

Figure 4, comprising Figures 4A and 4B depicts the effects of ATP hydrolysis or ADP binding by the hMSH2/hMSH6 heterodimer on mismatched DNA binding. Figure 4A is a graph depicting the results of gel mobility shift assays performed in the presence or absence of 15 micromolar ATP and in the presence or

5   absence of 15 micromolar ATP-γ-S. Magnesium chloride was added at the time designated "0", and samples of the assay mixture were collected at the indicated times (in minutes). The binding reaction in each mixture was halted by addition of 5 millimolar EDTA. Figure 4B is a graph depicting the results of gel mobility shift assays performed in the presence of the indicated (in millimolar) concentrations of ATP

10   or ADP or both.

Figure 5 comprises Figures 5A and 5B. Figure 5A is a graph which depicts the results obtained in the assays described herein for detecting the rate of a single round of ATP hydrolysis by the complex. Figure 5B is a graph which depicts the results obtained in assays described herein for detecting the rate of a single round of

15   ATP hydrolysis by the complex in the presence of selected amounts of mismatched DNA.

Figure 6, comprising Figures 6A, 6B, 6C, and 6D, depicts the results of experiments performed to assess the effects of ATP, homologous DNA, or both, on the dissociation of the hMSH2:hMSH6 heterodimer from DNA. Figure 6A is an image of

20   the results obtained from gel mobility shift assays in which heterodimer-bound mismatched DNA was incubated with ATP for the time indicated in the image. Figure 6B is an image of the results obtained from gel mobility shift assays in which heterodimer-bound mismatched DNA was incubated with ATP and a 400-fold excess of homologous DNA for the time indicated in the image. Figure 6C is an image of the

25   results obtained from gel mobility shift assays in which heterodimer-bound mismatched DNA was incubated with a 400-fold excess of homologous DNA for the time indicated in the image. Figure 6D is an image of the results obtained from gel mobility shift assays in which the heterodimer was incubated with homoduplex DNA probe for fifteen minutes at 37°C (Lane A), the assay mixture was cooled to 4°C, and a

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1,100-fold excess of unlabeled competitor homoduplex DNA was added (Lane B). In each of Figure 6A, 6B, 6C, and 6D, "-" indicates assay mixtures which did not comprise the heterodimer.

5   **Figure 7** is a diagram which depicts the model of the hMSH2:hMSH6 heterodimer association with and dissociation from mismatched duplex DNA described herein. The ADP-bound form of the heterodimer ("MSH$_2$"), which is shown in the center of the diagram, is competent to bind mismatched duplex DNA, as shown at the bottom of the diagram, but cannot diffuse from the mismatch site on the DNA. Mismatched DNA-bound complex is enabled to diffuse to a different position on the

10   DNA by displacement of the ADP molecule bound thereto by an ATP molecule (here indicated "*ATP"), which yields the ATP-bound form of the heterodimer. The ATP-bound form of the heterodimer is able to dissociate from a free end of the duplex DNA, but not from a blocked end of the duplex DNA. After dissociating from the duplex DNA, the ATP-bound form of the heterodimer is converted to the ADP-bound form by

15   hydrolysis of the heterodimer-bound ATP molecule, catalyzed by intrinsic ATPase activity of the heterodimer.

       Figure 8 lists the nucleotide sequence of single nucleotide chains of some of the 39- and 81-base pair DNA substrates described herein (SEQ ID NOS: 2, 3, 5, and 6).

20   **Figure 9**, comprising Figures 9A, 9B, 9C, and 9D, is a series of images, each of which depicts a whole mount view of an $Msh2^{-/-}p53^{-/-}$ embryo at day 11.5 of development. The embryo depicted in Figure 9A is a male $Msh2^{-/-}p53^{-/-}$ mouse embryo, and exhibits phenotypically normal embryonic development, relative to mice having the same genotypic background. The embryos depicted in Figures 9B, 9C, and

25   9D are female $Msh2^{-/-}p53^{-/-}$ mouse embryos that are littermates of the male mouse depicted in Figure 9A. The female mouse embryos depicted in Figures 9B, 9C, and 9D exhibit developmental arrest having a phenotype corresponding to that expected at day 9.5 of embryonic development.

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Figure 10, comprising Panels A, B, C, D, E, and F, is a series of
images, each of which depicts a paraffin embedded section obtained from an 11.5 day
old female mouse embryo. The images in Panels A, C, and E each depict a section
obtained from an 11.5 day old normal embryo. The images in Panels B, D, and F each

5   depict a section obtained from an 11.5 day old $Msh2^{-/-}p53^{-/-}$ mouse embryo. The
sections depicted in Panels A and B are at 100× magnification and are stained with
hematoxylin and eosin. Magnification of the normal embryo is of the somite region of
a sagittal section. The sections depicted in Panels C and D are at 100× magnification
and are chromogenically-TUNEL stained. The sections depicted Panels E and F are at

10   40× magnification and are fluorescently-TUNEL stained. Cells undergoing apoptosis
in normal female embryos were rare; chromogenically- and fluorescently-TUNEL
stained cells depicted in Panels C and E represent circumscribed apoptotic foci
normally found in developing mouse embryos.

Figure 11 is a graph which depicts Kaplan-Meier survival probabilities

15   of $Msh2^{-/-}$, $p53^{-/-}$, and $Msh2^{-/-}p53^{-/-}$ mice.

Figure 12 is a diagram which indicates the primary structure of
^{35}S-labeled IVTT-hMSH3 polypeptides used to identify approximate boundaries of
hMSH2-interaction regions of hMSH3. "Amino Acid Number" refers to the amino
acid residues of hMSH3 which the corresponding IVTT-hMSH3 polypeptide

20   :   comprised. The rectangular entities in the central part of the figure represent relative
positions of the amino acid residues which the corresponding IVTT-hMSH3
polypeptide comprised with respect to full length hMSH3, which is represented by
polypeptide 1). The symbol, Δ, indicates a deleted region of a polypeptide. The
shaded regions of polypeptide 1) represent the hMSH2-interaction regions of hMSH3.

25   "Interaction with hMSH2" indicates whether or not the corresponding polypeptide
interacted with GST-hMSH2.

Figure 13 is a diagram which indicates the primary structure of
^{35}S-labeled IVTT-hMSH2 polypeptides used to identify approximate boundaries of
hMSH3-interaction regions of hMSH2. "Amino Acid Number" refers to the amino

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acid residues of hMSH2 which the corresponding IVTT-hMSH2 polypeptide

comprised. The rectangular entities in the central part of the figure represent relative

positions of the amino acid residues which the corresponding IVTT-hMSH2

polypeptide comprised with respect to full length hMSH2, which is represented by

5   polypeptide 1). The shaded regions of polypeptide 1) represent the hMSH3-interaction

regions of hMSH2. "Interaction with hMSH3" indicates whether or not the

corresponding polypeptide interacted with GST-hMSH3.

Figure 14 is a diagram which indicates the primary structure of

^{35}S-labeled IVTT-hMSH2 polypeptides used to identify the linear orientation of the

10   hMSH3-interaction regions of hMSH2. "Amino Acid Number" refers to the amino

acid residues of hMSH2 which were present in the corresponding IVTT-hMSH2

polypeptide. The rectangular entities in the central part of the figure represent relative

positions of the amino acid residues which were present in the corresponding

IVTT-hMSH2 polypeptide with respect to full length hMSH3, which is represented by

15   polypeptide 1). The symbol, Δ, indicates a deleted region of a polypeptide.

"Interaction with specific hMSH3 domains" indicates whether or not the corresponding

polypeptide interacted with a GST-hMSH3 fusion protein comprising the amino-

terminal ("NH$_4$$^+$") interaction region of hMSH3 or with a GST-hMSH3 fusion protein

comprising the carboxy-terminal ("COO$^-$") interaction region of hMSH3.

20   Figure 15 is a diagram which indicates the primary structure of

^{35}S-labeled IVTT-hMSH6 polypeptides used to identify approximate boundaries of ·

hMSH2-interaction regions of hMSH6. "Amino Acid Number" refers to the amino

acid residues of hMSH6 which were present in the corresponding IVTT-hMSH2

polypeptide. The rectangular entities in the central part of the figure represent relative

25   positions of the amino acid residues which were present in the corresponding

IVTT-hMSH6 polypeptide with respect to full length hMSH6, which is represented by

polypeptide 1). The shaded regions of polypeptide 1) represent the hMSH2-interaction

regions of hMSH6. "Interaction with hMSH2" indicates whether or not the

corresponding polypeptide interacted with GST-hMSH2.

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Figure 16 is a diagram which indicates the primary structure of ^{35}S-labeled IVTT-hMSH2 polypeptides used to identify approximate boundaries of hMSH6-interaction regions of hMSH2. "Amino Acid Number" refers to the amino acid residues of hMSH2 which were present in the corresponding IVTT-hMSH2

5   polypeptide. The rectangular entities in the central part of the figure represent relative positions of the amino acid residues which the corresponding IVTT-hMSH2 polypeptide comprised with respect to full length hMSH2, which is represented by polypeptide 1). The shaded regions of polypeptide 1) represent the hMSH6-interaction regions of hMSH2. "Interaction with hMSH3" indicates whether or not the

10   corresponding polypeptide interacted with GST-hMSH6.

Figure 17 is a diagram which indicates the primary structure of ^{35}S-labeled IVTT-hMSH2 polypeptides used to identify the linear orientation of the hMSH6-interaction regions of hMSH2. "Amino Acid Number" refers to the amino acid residues of hMSH2 which were present in the corresponding IVTT-hMSH2

15   polypeptide. The rectangular entities in the central part of the figure represent relative positions of the amino acid residues which were present in the corresponding IVTT-hMSH2 polypeptide with respect to full length hMSH6, which is represented by polypeptide 1). The symbol, Δ, indicates a deleted region of a polypeptide. "Interaction with specific hMSH6 domains" indicates whether or not the corresponding

20   polypeptide interacted with a GST-hMSH6 fusion protein comprising the amino-terminal ("NH_4^+") interaction region of hMSH6 or with a GST-hMSH6 fusion protein comprising the carboxy-terminal ("COO^-") interaction region of hMSH6.

Figure 18 is a diagram which illustrates a model of hMSH2 consensus interaction with hMSH3 or hMSH6. The interaction regions of hMSH2, hMSH3, and

25   hMSH6 are indicated in gray and are connected with lines that illustrate the specificity of each region to its corresponding interaction partner region. The nucleotide binding regions of hMSH2, hMSH3, and hMSH6 are indicated as black boxes. The location of HNPCC-associated mutations tested in these studies are illustrated as black diamonds.

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Figure 19, comprising Figures 19A, 19B, and 19C, lists the nucleotide sequence of cDNA encoding hMSH5 (SEQ ID NO: 30) and the putative amino acid sequence of hMSH5 (SEQ ID NO: 29).

DETAILED DESCRIPTION OF THE INVENTION

5   The invention relates to a method of binding one or more MutS homolog (MSH) dimers to a mismatched duplex DNA. The invention also relates to methods of using adenine nucleotides to modulate recognition of mismatched duplex DNA and to modulate DNA-associated diffusion of MSH dimers after binding of such dimers to mismatched duplex DNA. The invention further relates to a method of

10   binding a complex comprising a MutL homolog and a MutS homolog to mismatched duplex DNA. The MutL homolog interacts with the MutS homolog and influences the ability of the MutS homolog to bind with a mismatched region of the duplex DNA.

A Summary of Some of the Novel Properties of MutS Homologs and MutL Homologs

The compositions, kits, and methods of the invention may be better

15   understood by understanding the novel properties of MutS homologs and MutL homologs which have been discovered by the inventors. This section presents merely a brief introduction to several these properties. It is understood that the operability of the compositions, kits, and methods of the invention does not depend upon the correctness of the information provided in this section.

20   An important aspect of the invention is the discovery that MutS homolog (MSH) dimers and, in some organisms, MSH heterodimers, associate with mismatched regions of a mismatched duplex DNA. Binding of a MutS dimer to mismatched DNA occurs when ADP, but not ATP, is bound to the MSH dimer. The MSH dimer may, for example, be in the form of an MSH homodimer (e.g. an *E. coli*

25   MutS dimer) or an MSH heterodimer (e.g. a human MSH heterodimer such as an hMSH2:hMSH3 dimer, an hMSH2:hMSH6 dimer, or an hMSH4:hMSH5 dimer). This association may be effected either *in vitro* or *in vivo*.

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ADP-bound MSH dimer associated with a mismatched region of a mismatched duplex DNA does not move along the duplex DNA, but instead remains located at the mismatched region. Exchange of ATP for the ADP bound to the MSH dimer confers to the MSH dimer DNA-associated diffusibility, which means that the

5   MSH dimer becomes able to move from the site of the mismatched region of the duplex DNA to another site on the same duplex DNA. If the mismatched duplex DNA has a free end, then the DNA-associated diffusibility of an ATP-bound MSH dimer enables the dimer to the duplex DNA dissociate from the duplex DNA. If the mismatched duplex DNA does not have a free end (e.g. the DNA is circular or has

10   bulky moieties such as proteins bound to the ends thereof), then neither the ADP-bound form or the ATP-bound form of the MSH dimer is able to dissociate from the duplex DNA.

Because MSH heterodimers, in their ATP-bound form, exhibit DNA-associated diffusibility with regard to the duplex DNA with which they are associated,

15   an ATP-bound MSH dimer will not necessarily be associated with the mismatched region of a mismatched duplex DNA, but instead may have diffused away from the mismatched region to complementary region of the same mismatched duplex DNA. Thus, a mismatched duplex DNA having one or more ATP-bound MSH dimers associated therewith is able to associate with another MSH dimer in an ADP-bound

20   form. Therefore, numerous MSH dimers may be associated with a mismatched duplex DNA by contacting the DNA with ADP-bound MSH dimers in the presence of a binding solution which comprises ATP. It is understood that certain MSH homodimers (e.g. hMSH2 dimers; Fishel et al., 1994, Science 266:1403-1405) exhibit little or no alteration in activity associated with adenine nucleotide binding, and may be useful for

25   these properties. For example, hMSH2 binds a variety of mismatched nucleotides but remains unperturbed in the presence of either ADP or ATP (Fishel et al., 1994, Science 266:1403-1405).

MSH dimers exhibit an intrinsic ATP hydrolytic activity, and this hydrolytic activity is greatly enhanced in their non-DNA-associated form, but not in

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their DNA-associated form. Thus, an ATP-bound MSH dimer associated with DNA remains ATP-bound. However, ATP bound to an MSH dimer is rapidly converted to ADP if the dimer is not associated with DNA. Thus, the intrinsic ATPase activity exhibited by MSH dimers catalyzes the transformation of an ATP-bound dimer (which

5   cannot associate with a mismatched region of DNA) to an ADP-bound dimer (which can associate with a mismatched region of DNA). In addition, the mismatched DNA-associated form of MSH dimers are able to more rapidly exchange ATP in place of ADP bound to the dimer than MSH dimers not associated with DNA or associated with non-mismatched DNA.

10   Without wishing to be bound by any particular theory of operation, binding of MSH dimers to mismatched duplex DNA may be visualized as illustrated in Figure 7. An ADP-bound MSH dimer associates with the mismatched region of the DNA. Exchange of ATP in place of the ADP bound to the MSH dimer enables the dimer to diffuse to a different position on the DNA. The DNA-associated ATP-bound

15   MSH dimer cannot dissociate from a blocked end of the DNA in the presence of Mg^{2+}, but can dissociate from a free end of the DNA. Alternately, ATP-bound MSH dimer can be dissociated from DNA which does not have a free end in the presence of EDTA or a high salt concentration. ATP-bound MSH dimer not associated with DNA is able to hydrolyze the ATP moiety, yielding an ADP-bound MSH dimer, which is then able

20   to associate with a mismatched region of DNA.

An MSH dimer may be thought of as 'molecular switch,' wherein the ADP-bound dimer represents an 'ON' state, and wherein the ATP-bound dimer represents an 'OFF' state. In the 'ON' state, the dimer is able to associate with a mismatched region of DNA but is not able to diffuse to a different position on the DNA

25   with which it is associated. In the 'OFF' state, the dimer is not able to associate with a mismatched region of DNA but is able to diffuse to a different position on the DNA with which it is associated. Recalling the involvement of MutS homologs in DNA mismatch repair and, as demonstrated herein, in control of the cell replication cycle, it is understood that compounds which modulate the transition of MSH dimers from the

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'ON' to the 'OFF' state or *vice versa* may be used to modulate DNA mismatch repair, timing of and progression through the cell replication cycle, and/or the physiological process(es) associated with either DNA mismatch repair or the cell replication cycle.

A MutL homolog improve the intrinsic ATPase activity exhibited by a MSH dimer when the MutL homolog associates with the MSH dimer. MutL homologs may thus be analogized to GTPase accelerating proteins (sometimes designated "GAP proteins") which have been described in the context of G protein activity. Without wishing to be bound by any particular theory, it is thought that association of a MutL homolog with a MSH dimer increases the rate of dissociation of the ATP-bound MSH dimer from duplex DNA and increases the rate at which ATP is converted to ADP by the non-duplex DNA-associated ATP-bound MSH dimer, thereby rendering the MSH dimer able to bind to a mismatched duplex DNA more rapidly than in the absence of the MutL homolog.

The biochemical properties of MutS homologs and MutL homologs described in this section are used advantageously in the compositions, kits, and methods of the invention.

Definitions

As used herein, each of the following terms has the meaning associated with it in this section.

A "MutS homolog" is a protein which comprises a region which exhibits significant sequence similarity with at least one of the following regions of the human MSH2 protein (wherein the regions are indicated by the numbers of the amino acid residues of MSH2 which, inclusively, bound the region; the corresponding amino acid sequences of hMSH2 is indicated thereafter in parentheses):

Region I: hMSH2 amino acid residues 37-57 (LFDRGDFYTA HGEDALLAAR E; SEQ ID NO: 24);

Region II: hMSH2 amino acid residues 336-368 (TPQGQRLVNQ WIKQPLMDKN RIEERLNLVE AFV; SEQ ID NO: 25);

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Region III: hMSH2 amino acid residues 635-662 (LKASRHACVE

VQDEIAFIPN DVYFEKDK; SEQ ID NO: 26);

Region IV: hMSH2 amino acid residues 667-770 (IITGPNMGGK

STYIRQTGVI VLMAQIGCFV PCESAEVSIV DCILARVGAG DSQLKGVSTF

MAEMLETASI LRSATKDSLI IIDELGRGTS TYDGFGLAWA ISEY; SEQ ID NO:

27); and

Region V: hMSH2 amino acid residues 812-852 (LTMLYQVKKG

VCDQSFGIHV AELANFPKHV IECAKQKALE L; SEQ ID NO: 28).

The amino acid sequence of hMSH2 has been described (e.g. Fishel et al., 1993, Cell

75:1027). Preferably, the MutS homolog of the invention comprises a region which

exhibits significant sequence similarity with Region IV, and more preferably with both

Region IV and Region V. It is also preferred that the MutS homolog comprises a

plurality of regions, each of which exhibits significant sequence similarity with one of

Regions I-V of hMSH2, and more preferred that the MutS homolog comprises regions

which independently exhibit significant sequence similarity with each of Regions I-V

of hMSH2. Thus, MutS homologs which are included in the invention include, but are

not limited to *Aquifex aeolicus* MutS, *Aquifex aeolicus* MSH, *Aquifiex pyrophilicus*

MutS, *Arabidopsis thaliana* MSH2, *Arabidopsis thaliana* MSH6, *Azotobacter*

vinelandii MutS, *Bacillus subtilis* MutS, *Bacillus subtilis* MSH, *Caenorhabdis elegans*

MSH4, *Caenorhabdis elegans* MSH5, *Drosophila melanogaster* MSH2, *Escherichia*

coli MutS, *Homo sapiens* MSH2, *Homo sapiens* MSH3, *Homo sapiens* MSH4, *Homo*

sapiens MSH5, *Homo sapiens* MSH6, *Haemophilus influenzae* type B MutS,

Helicobacter pylori MSH, *Mus musculus* MSH2, *Mus musculus* MSH3, *Mus musculus*

MSH6, *Neurospora crassa* MSH2, *Rattus norvegicus* MSH2, *Saccharomyces*

cerevisiae MSH1, *Saccharomyces cerevisiae* MSH2, *Saccharomyces cerevisiae* MSH3,

Saccharomyces cerevisiae MSH4, *Saccharomyces cerevisiae* MSH5, *Saccharomyces*

cerevisiae MSH6, *Saccharomyces pombe* MSH1, *Saccharomyces pombe* MSH2,

Saccharomyces pombe Swi4, *Saccharomyces pombe* MutS, *Salmonella typhimurium*

MutS, *Synechocystis* sp. MutS, *Synechocystis* sp. MSH, *Thermus aquaticus* MutS,

Thermotoga maritima MutS, and *Thermus thermophilus* MutS, each of which proteins is described either herein or in the prior art.

A "MutL homolog" is a protein which exhibits significant similarity to the MutL protein of *E. coli*. MutL homologs include, but are not limited to, eukaryotic MLH1, MLH2, PMS1, and PMS2 proteins.

A protein or a region of a protein exhibits "significant similarity" to another protein or a region of another protein if, when the two proteins or regions are compared in a selected alignment, at least 50%, at least 70%, at least 85%, at least 95%, or at least 99% of the aligned amino acid residues of the two proteins or the two regions are either identical or similar. Similar amino acid residues are indicated by the groups listed on the following lines:

glycine, alanine;

valine, isoleucine, leucine;

aspartic acid, glutamic acid;

asparagine, glutamine;

serine, threonine;

lysine, arginine; and

phenylalanine, tyrosine.

A "heterodimer" is a protein which comprises more than one subunit, wherein at least one subunit has an amino acid sequences which is different from the amino acid sequence of another subunit of the same protein. Heterodimers having an 'A' protein subunit and a 'B' protein subunit are herein designated "A:B heterodimers".

A "DNA strand" is a single polydeoxyribonucleotide.

A "duplex DNA" is a molecule that comprises at least one polydeoxyribonucleotide, wherein at least a portion of the polydeoxyribonucleotide has a double-stranded, hydrogen bonded conformation.

A "mismatched" duplex DNA is a duplex DNA wherein at least one DNA strand comprises a region which has at least one nucleotide residue that is not base-paired with a complementary nucleotide residue and which is flanked by regions

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wherein at least about ten nucleotide residues are all base-paired with complementary nucleotide residues.

A first region of an DNA "flanks" a second region of the DNA if the two regions are adjacent one another or if the two regions are separated by no more than about 10 nucleotide residues, and preferably no more than 1 nucleotide residue.

A "non-mismatched" duplex DNA is a duplex DNA wherein all nucleotide residues of the double-stranded portion thereof are base-paired with complementary nucleotide residues.

"Complementary" refers to the broad concept of sequence complementarity between regions of two nucleic acid strands or between two regions of the same nucleic acid strand. It is known that an adenine residue of a first nucleic acid region is capable of forming specific hydrogen bonds ("base-pairing") with a residue of a second nucleic acid region which is antiparallel to the first region if the residue is thymine or uracil. Similarly, it is known that a cytosine residue of a first nucleic acid strand is capable of base pairing with a residue of a second nucleic acid strand which is antiparallel to the first strand if the residue is guanine. A first region of a nucleic acid is complementary to a second region of the same or a different nucleic acid if, when the two regions are arranged in an antiparallel fashion, at least one nucleotide residue of the first region is capable of base pairing with a residue of the second region. Preferably, the first region comprises a first portion and the second region comprises a second portion, whereby, when the first and second portions are arranged in an antiparallel fashion, at least about 50%, and preferably at least about 75%, at least about 90%, or at least about 95% of the nucleotide residues of the first portion are capable of base pairing with nucleotide residues in the second portion. More preferably, all nucleotide residues of the first portion are capable of base pairing with nucleotide residues in the second portion.

A chemical entity such as a molecule is "bound" to another chemical entity if at least one portion of each of the two chemical entities are covalently or non-covalently bonded to one another in an essentially fixed position. By way of example,

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as described herein, an ADP-bound form of an MSH dimer is bound to a mismatched region of a duplex DNA because the MSH dimer predominantly associates with the duplex DNA at the location of the mismatch.

A chemical entity such as a molecule is "associated" with another

5   chemical entity if at least one of the chemical entities can change its position relative to the other without becoming dissociated therefrom. By way of example, as described herein, an ATP-bound form of an MSH dimer is associated with a mismatched duplex DNA because the MSH dimer can diffuse to a different position on the DNA without dissociating therefrom.

10   A duplex DNA is "modified" if a chemical entity such as a molecule is bound to, associated with, or dissociated from the duplex DNA, or if the duplex DNA is segregated from a population of DNA molecules.

A duplex DNA has a "free end" if the duplex DNA is not circular and if both ends of the duplex DNA are not blocked.

15   An end of a duplex DNA is "blocked" if a bulky moiety is bound to a portion of the duplex DNA between a reference point on the duplex DNA and the end of the duplex DNA.

A "bulky moiety" bound to a portion of a duplex DNA is any chemical entity which has a size sufficient to prevent sliding of an ATP-bound MSH dimer along

20   the DNA duplex from a location on one side of the bulky moiety to a location on the other side of the bulky moiety. Examples of bulky moieties include proteins, metallic, glass, or polymeric surfaces, and the like.

A "gamma-modified ATP analog" is an ATP molecule which has an a group attached to the gamma phosphodiester moiety thereof, whereby the beta-gamma

25   phosphodiester linkage is cleaved by an MSH dimer with an efficiency less than 25% of the efficiency with which ATP is hydrolyzed by the MSH dimer. By way of example, ATP-γ-S is a gamma-modified ATP analog.

A "gamma-hydrolysis-resistant ATP analog" is an ATP molecule which has an altered beta-gamma phosphodiester linkage chemistry whereby the altered beta-

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gamma phosphodiester linkage cannot be cleaved be either the intrinsic ATP hydrolytic activity of an MSH dimer or by the ATP hydrolytic activity of an MSH dimer-MutL homolog complex. Examples of gamma-hydrolysis-resistant ATP analogs include, but are not limited to ATP-PNP and ATP-PCP, which are compounds well known and described in the art.

A solution is "substantially free" of ATP when the concentration of ATP is very low (e.g. less than 30 nanomolar, and preferably less than 1 nanomolar).

The term "substantially pure" describes a compound, e.g., a protein or polypeptide which has been separated from components which naturally accompany it. Typically, a compound is substantially pure when at least 10%, more preferably at least 20%, more preferably at least 50%, more preferably at least 60%, more preferably at least 75%, more preferably at least 90%, and most preferably at least 99% of the total material (by volume, by wet or dry weight, or by mole percent or mole fraction) in a sample is the compound of interest. Purity can be measured by any appropriate method, e.g., in the case of polypeptides by column chromatography, gel electrophoresis or HPLC analysis. A compound, e.g., a protein, is also substantially purified when it is essentially free of naturally associated components or when it is separated from the native contaminants which accompany it in its natural state.

"Nullizygous" refers to an animal which possesses a pair of null mutant alleles at a given genetic locus. Hence, a nullizygous Xxx mouse (wherein Xxx is any gene normally present in a mouse) does not possess a functional Xxx gene, whereas a wild-type mouse may possess one or two functional copies of the Xxx gene. To illustrate the notation used herein, the term "nullizygous Xxx mouse" is synonymous with the term "$Xxx^{-/-}$ mouse." Similarly, a "heterozygous Xxx mouse" has one functional Xxx allele and one non-functional Xxx allele, and is synonymous with the term "$Xxx^{+/-}$ mouse." A "wild type mouse" has at least one copy, and possibly two copies, of a functional Xxx allele, and is synonymous with the term "$Xxx^{+/\pm}$ mouse." A "homologous wild type mouse' has two copies of a functional Xxx allele, and is synonymous with the term "$Xxx^{+/+}$ mouse."

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As used herein, an "instructional material" includes a publication, a recording, a diagram, or any other medium of expression which can be used to communicate the usefulness of the compositions and methods of the invention for associating a MSH dimer with a mismatched duplex DNA. The instructional material

5   of the kit of the invention may, for example, be affixed to a container which contains the dimer or be shipped together with a container which contains the dimer. Alternatively, the instructional material may be shipped separately from the container with the intention that the instructional material and the dimer be used cooperatively by the recipient.

10   A solution comprises "high salt" if the concentration of one or more salts in the solution is, cumulatively, at least about 1 molar, preferably at least about 3 molar.

A "double-stranded DNA-cleaving enzyme" is an enzyme which catalyzes hydrolysis of both strands of a duplex DNA, leaving either blunt or staggered

15   ends. Examples of double-stranded DNA-cleaving enzymes include, but are not limited to, restriction endonucleases.

Description

The invention relates to a method of modifying a mismatched duplex DNA. The method comprises contacting a MutS homolog (MSH) dimer and the

20   mismatched duplex DNA in the presence of a binding solution. The binding solution comprises either ADP and ATP, and the concentration of ATP in the binding solution is less than about 3 micromolar, preferably less than about 0.3 micromolar, and more preferably wherein the binding solution is substantially free of ATP. Alternately, ADP is used in the absence of ATP, or at least in excess with respect to ATP (i.e. ADP at a

25   2-fold, 10-fold, or 100-fold greater excess relative to ATP). The MSH dimer thereby binds ADP. When the ADP-bound MSH dimer is contacted with the mismatched duplex DNA, the dimer associates with the mismatched region of the DNA, thus forming a modified mismatched duplex DNA.

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The MSH dimer may be a homodimer or a heterodimer of any MutS homolog which is presently known to comprise or is discovered to comprise one or more which exhibits significant sequence similarity with at least one of Region I-V of human MSH2 (hMSH2), as described herein. The MutS homolog may be a

5   prokaryotic MutS homolog or a eukaryotic MutS homolog. Preferably, the MutS homolog is a heterodimer, more preferably a heterodimer comprising MutS homologs obtained from a single species of organism. Thus, by way of example, the MSH dimer useful in the methods, kits, and compositions of the invention may be the *E. coli* MutS protein, an hMSH2 homodimer, a heterodimer comprising hMSH2 and either hMSH3

10   or hMSH6, a heterodimer comprising hMSH4 and hMSH5, a yeast MSH2 protein homodimer, a heterodimer comprising yeast MSH2 and either yeast MSH3 or yeast MSH6, a homodimer of a rat MSH2 (e.g. GenBank accession number X93591), a dimer of a *Xenopus* homolog of hMSH2 (Varlet et al., 1994, Nucl. Acids Res. 22:5723-5728), a homodimer of *Drosophila* MSH2 (e.g. GenBank accession number U17893), a

15   homodimer of murine MSH2 (e.g. GenBank accession number X93591, Varlet et al., 1994, Nucl. Acids Res. 22:5723-5728), a heterodimer comprising murine MSH2 and either murine MSH3 (e.g. Rep-3; Linton et al., 1989, Mol. Cell. Biol. 9:3058-3072; Smith et al., 1990, Mol. Cell. Biol. 10:6003-6012) or murine MSH6 (e.g. Gen Bank accession number U42190), and the like. The MutS homolog of the MSH dimer used

20   in the compositions, kits, and methods of the invention may also be any of the 41 MutS homologs and presently listed in the NCBI database. It is understood that, given the high degree of similarity among mammalian MutS homologs (Fishel et al., 1997, Curr. Op. Genet. Develop. 7:105-113), a dimer of any mammalian hMSH2 homolog can be used in the methods of the invention.

25   The mismatched duplex DNA molecule useful in the methods of the invention may be any duplex DNA molecule having at least one mismatched region. By way of example, the DNA molecule may be a linear DNA molecule, a circularized DNA molecule such as a plasmid or a viral genome, a chromosome, a cDNA generated by reverse transcription of an RNA molecule, a PCR primer, a PCR product, a complex

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formed between a single-stranded DNA probe and another single-stranded DNA molecule, and the like. The mismatched region may be any region of a duplex DNA molecule in which the two DNA strands of the molecule are not completely complementary. By way of example, the mismatched region may comprise one or

5   more pairs of mismatched nucleotides in an otherwise complementary region of a duplex DNA molecule, a region of a duplex DNA molecule wherein a thymine dimer exists on one DNA strand of the molecule, a region of a duplex DNA molecule comprising a nucleotide which has been covalently modified by an agent capable of reacting with a nucleotide, such as cisplatin, a region of a duplex DNA molecule which

10   comprises an alkyl-O-6-methyl guanine residue, a region of a duplex DNA molecule which comprises a single stranded loop of one or more nucleotides, a region of a duplex DNA molecule which comprises a pyrimidine dimer, and the like.

       While any amount of ADP can be used in the binding solution of the method of the invention, it is preferred that the homolog be contacted with the

15   mismatched duplex DNA in the presence of a binding solution comprising at least about 100 nanomolar ADP, preferably at least about 6 micromolar ADP, and more preferably at least about 60 micromolar ADP. As described with greater particularity in Example 1, ATP displaces ADP from the MSH dimer when the dimer is associated with a mismatched region of duplex DNA. Thus, it is important either that the

20   concentration of ATP in the solution be minimized, for example by maintaining the concentration of ATP lower than about 3 micromolar, preferably lower than about 0.3 micromolar, and more preferably lower than about 10 nanomolar, or that the ratio of the concentration of ADP in the solution to the concentration of ATP in the solution be greater than a minimum value, such as about two, and preferably greater than about

25   eight, and even more preferably greater than about sixteen. Preferably, the solution is substantially free of ATP or the ratio of ADP to ATP is much greater than sixteen (e.g. [ADP]:[ATP] is 100:1 or greater).

       It is understood that gamma-hydrolysis-resistant ATP analogs, certain other ATP analogs, and other ADP analogs may be bound to an MSH dimer, and that

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these analog-bound dimers will associated with mismatched duplex DNA. By way of example, MSH2:MSH6 dimers will associate with mismatched DNA in the presence of either ATP-PNP or ATP-PCP.

The MSH dimer that is useful in the compositions, kits, and methods of the invention may be used in a variety of states of purity or isolation. For example, the dimer may be present in a liquid which a variety of other proteins, nucleic acids, lipids, single stranded nucleic acids, non-mismatched duplex DNA, and the like, it being understood that if the dimer is used in the form of a mismatched duplex DNA-containing liquid then it may be necessary to dissociate, and possibly to separate, the dimer from the mismatched DNA prior to using it in the compositions, kits, and methods of the invention. Preferably, the MSH dimer is substantially purified.

In many of the compositions, kits, and methods of the invention, the MSH dimer or the mismatched duplex DNA may bound to a support. Furthermore, each of the MSH dimer and the mismatched duplex DNA may be bound to different supports.

The MSH dimer or a MutS homolog of the dimer may be bound to a support using any known method for attaching a protein to a surface. For example the MutS homolog may be bound to a support by way of an antibody which is covalently bound to the support and which has a variable region which specifically binds to the MutS homolog. By way of example, an antibody which specifically binds to hMSH2 such as the antibody described by Kinzler et al. (PCT publication number WO96/41192) may be used to bind an hMSH2 protein dimer or a complex comprising an hMSH2 protein molecule and either an hMSH3 protein molecule or an hMSH6 protein molecule to a support to which the antibody is fixed. Methods of fixing an antibody to a support have been described in the art (e.g. Harlow et al., 1988, Antibodies: A Laboratory Manual, Cold Spring Harbor, New York). Alternately, covalent, ionic, hydrophobic, or other types of bonding forces may be used to attach an MSH dimer or a MutS homolog to a support.

The duplex DNA molecule may be bound to a support using any known method for attaching a nucleic acid to a support. By way of example, the nucleic acid may be covalently linked to a biotin molecule and the support may be linked to or coated with a streptavidin molecule, whereby the streptavidin molecule is capable of

5   binding the biotin molecule, thereby linking the nucleic acid to the support. Further by way of example, the duplex DNA may be covalently attached to a chemical substituent present on a surface of the support. Alternately, covalent, ionic, hydrophobic, or other types of bonding forces may be used to attach the duplex DNA to the support.

Supports to which an MSH dimer, a MutS homolog, or a duplex DNA

10   molecule may be bound include any support known in the art for use in *in vitro* or *in vitro* biochemical or medical applications. By way of example, and not limitation, such supports include latex and other polymeric beads, particles, plates, supports, chromatography media, implants, drug delivery vehicles, metal and glass surfaces, gelatinous surfaces such as agarose, alginates, and polyacrylamides, and the like. It is

15   important that the ability of the MutS homolog monomers or MSH dimers which are bound to the support be attached in such a way that the ability of the monomers to dimers to attain altered conformations is not significantly hindered. It is understood that, for example, by isolating antibodies which specifically bind to various epitopes on the monomer or dimer surface, a variety of antibodies may be isolated an used to bind

20   monomers or dimers to a support. By assaying the ability of the support-bound monomers or dimers to bind to mismatched DNA in the presence of ADP, for example as described herein, an antibody or other support which attaches the monomers or dimers to a support without hindering their ability to bind mismatched duplex DNA may be identified. Such methods are routine in the art of protein immobilization and

25   are not further described herein.

As disclosed herein, after an ADP-bound MSH dimer binds to a mismatched region of a duplex DNA, exchange of ATP for the ADP bound to the dimer results in release of the dimer from the mismatched region, whereby the ATP-bound dimer is enabled to diffuse to a different position on the DNA. If the dimer is

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able to diffuse to a free end of the duplex DNA, the dimer may dissociate from the duplex DNA.

If the duplex DNA does not have a free end, then ATP-bound dimer may diffuse away from the mismatched region of the duplex DNA, but may not

5 dissociate from the DNA. Thus, if the duplex DNA does not have a free end, a plurality of copies of the dimer may be associated with the DNA in the presence of ATP. No upper limit is known for the number of dimers which may be associated with the DNA, but it is contemplated that this number is roughly proportional to the length of the duplex DNA. It is understood that association of multiple copies of an MSH

10 dimer with a mismatched duplex DNA may be advantageous in situations in which association of the dimer with the DNA is to be detected. Multiple copies of the dimer may boost the detection limit of the DNA to be detected, increasing the signal-to-noise ratio of the detection method.

Duplex DNA not having a free end may be circular DNA or it may be

15 linear DNA wherein both ends of the DNA are blocked. Ends of duplex DNA may be blocked by binding bulky moieties such as proteins to the DNA either directly (e.g. by covalently attaching the protein to the DNA or by binding the protein to the DNA non-covalently with high affinity) or via a linker (e.g. by biotinylating the DNA and binding an avidin such as streptavidin to the biotin moiety). Bulky moieties which may be used

20 to block the ends of duplex DNA include, but are not limited to, proteins, supports, hairpin DNA structures, stem-and-loop DNA structures, and multiple a stem-and-loop DNA structures. Association of MSH dimers with DNA having one, two, or no free ends is expressly contemplated.

The mismatched duplex DNA to which an MSH dimer is to be bound

25 may, for example, comprise a first DNA strand having a reference nucleotide sequence and a second DNA strand selected from the group consisting of a DNA strand obtained from an organism, a DNA strand obtained by amplification of at least a portion of a polynucleotide obtained from an organism, a DNA strand obtained by cleavage of a polynucleotide obtained from an organism, and a DNA strand obtained by reverse

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transcription of a polynucleotide obtained from an organism. By way of example, the second DNA strand may comprise at least a portion of a gene associated with a cancer in the organism. This gene may, for example, be any of a number of oncogenes and tumor suppressor genes which are known in the art. Examples of such genes include,

5   for example, *abl, akt2, apc, bcl2α, bcl2β, bcl3, bcr, brca1, brca2, cbl, ccnd1, cdk4, crk-II, csf1r/fms, dbl, dcc, dpc4/smad4, e-cad, e2f1/rbap, egfr/erbb-1, elk1, elk3, eph, erg, ets1, ets2, fer, fgr/src2, fli1/ergb2, fos, fps/fes, fra1, fra2, fyn, hck, hek, her2/erbb-2/neu, her3/erbb-3, her4/erbb-4, hras1, hst2, hstf1, ink4a, ink4b, int2/fgf3, jun, junb, jund, kip2, kit, kras2a, kras2b, lck, lyn, mas, max, mcc, met, mlh1, mos, msh2, msh3,*
10   *msh6, myb, myba, mybb, myc, mycl1, mycn, nf1, nf2, nras, p53, pdgfb, pim1, pms1, pms2, ptc, pten, raf1, rb1, rel, ret, ros1, ski, src1, tal1, tgfbr2, thra1, thrb, tiam1, trk, vav, vhl, waf1, wnt1, wnt2, wt1,* and *yes1.* These genes are described in various publicly available databases, including the U.S. National Cancer Institute/National Center for Biotechnology Information Cancer Genome Anatomy Project database.
15   Various accession numbers for these genes are listed in Table 1.

Table 1

Gene Symbol	Entrez Accession	PubMed UID	UniGene CID	CGAP
ABL	X16416	90082420	Hs.82576	AA601510
AKT2	M95936	93028445	Hs.37433	AA505663
APC	M74088	91335210	Hs.75081	AA592971
BCL2ALPHA	M13994	86259760	Hs.89534	AA577385
BCL2BETA	M13995	86259760	Hs.99916	
BCL3	M31732	90199880	Hs.31210	AA527996
BCR	Y00661	85240564	Hs.2557	AA592930
BRCA1	U14680	95025896	Hs.66746	AA484941
BRCA2	X95161	96112016	Hs.34012	AA215820
CBL	X57110	92228506	Hs.99980	

	CCND1	M64349	91235304	Hs.82932	AA592929
	CDK4	U37022	8528263	Hs.95577	AA483705
	CRK-II	D10656	92334347	Hs.16	
	CSF1R/FMS	X03663	86175013	Hs.75116	AA595091
5	DBL	X12556	89052660	Hs.89543	
	DCC	X76132	95011532	Hs.68149	
	DPC4/SMAD4	U44378	96144684	Hs.75862	AA576881
	E-CAD	Z13009	93211394	Hs.82004	AA603448
	E2F1/RBAP	M96577	92346720	Hs.89494	
10	EGFR/ERBB-1	X00588	84219729	Hs.77432	AA587386
	ELK1	M25269	89203250	Hs.1399	AA576028
	ELK3	Z36715	95047310		AA262193
	EPH	M18391	88070650	Hs.1113	
	ERG	M17254	87263429	Hs.70388	
15	ETS1	X14798	89083219		
	ETS2	J04102	89042086	Hs.85146	AA480196
	FER	J03358	89261786		AA534773
	FGR(SRC2)	M12502	85205090	Hs.1422	
	FLI1/ERGB2	M98833	93075640	Hs.736	
20	FOS	V01512	83221560	Hs.25647	AA514238
	FPS/FES	X06292	86055727	Hs.7636	
	FRA1	X16707	90191709	Hs.4245	
	FRA2	X16706	90191709	Hs.89765	AA601534
	FYN	M14333	86287278	Hs.75390	AA524156
25	HCK	M16591	87257942	Hs.77058	
	HEK	M83941	92179233		
	HER2/ERBB-2/NEU	X03363	86118663	Hs.46254	AA508596
	HER3/ERBB-3	M29366	90083234	Hs.82186	AA570304
	HER4/ERBB-4	L07868	93189574	Hs.1939	

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	HRAS1	V00574	83141783	Hs.37003	AA483837
	HST2	X63454	92195660		
	HSTF1	J02986	87204251	Hs.1755	
	INK4A	L27211	94081956	Hs.1174	AA557137
5	INK4B	L36844	94359613		
	INT2/FGF3	X14445	89239468	Hs.37092	AA525331
	JUN	J04111	89057892	Hs.78465	AA582267
	JUNB	M29039	90090625	Hs.89792	AA503220
	JUND	X56681	91232849	Hs.2780	AA533575
10	KIP2	D64137	96209909	Hs.9039	AA524076
	KIT	X06182	88111521	Hs.81665	AA552932
	KRAS2A	L00045	83271513		
	KRAS2B	X01669	85087906		
	LCK	X13529	89123626	Hs.1765	AA282059
15	LYN	M16038	87172710	Hs.80887	AA524487
	MAS	M13150	86218084	Hs.99900	
	MAX	M64240	91173288	Hs.89500	AA592936
	MCC	M62397	91164855	Hs.1345	
	MET	J02958	87317655	Hs.35379	
20	MLH1	U07343	8145827	Hs.57301	
	MOS	J00119	82275068		
	MSH2	U04045	94084796	Hs.78934	AA502616
	MYB	M15024	87092302	Hs.1334	AA535078
	MYBA	X66087		Hs.2537	AA459003
25	MYBB	X13293	89083548	Hs.74605	AA603093
	MYC	X00196	84131953	Hs.79070	
	MYCL1	M19720	88094386	Hs.92137	
	MYCN	Y00664	88202932	Hs.25960	AA548970
	NF1	M89914	90335969	Hs.37170	AA534609

	NF2	L11353	93201601	Hs.902	AA617825
	NRAS	X02751	85269641	Hs.82602	AA558915
	P53	K03199	85267676	Hs.1846	AA514357
	PDGFB	M12783	87217119	Hs.1976	
5	PIM1	M27903	90382681	Hs.81170	AA251525
	PTC	U59464	8658145	Hs.54503	
	RAF1	X03484	86120351	Hs.85181	AA578685
	RB1	M15400	87149066	Hs.75770	AA594282
	REL	X75042	89330980	Hs.44313	AA279536
10	RET	M16029	87257826	Hs.6253	
	ROS1	M34353	90280463	Hs.1041	
	SKI	X15218	89345144	Hs.2969	AA258011
	SRC1	M16243	87257903	Hs.65442	AA523427
	TAL1	M29038	90099309	Hs.73828	AA551582
15	TGFBR2	M85079	92154690	Hs.82028	AA515322
	THRA1	Y00479	88067793	Hs.724	AA602782
	THRB	X04707	87090375		AA577807
	TIAM1	X86351	96129318	Hs.3205	
	TRK	M23102	89181575	Hs.85844	
20	VAV	X16316	90005432		
	VHL	L15409	93262488	Hs.78160	
	WAF1	L25610	94061996	Hs.74984	AA614342
	WNT1	X03072	86055728		
	WNT2	X07876	89005063	Hs.89791	AA601910
25	WT1	X51630	90158822	Hs.1145	
	YES1	M15990	87172733	Hs.75680	AA502695

In a preferred embodiment, the gene associated with a cancer is a gene associated with hereditary non-polyposis colon cancer. For example, the gene may be

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selected from the group consisting of *mlh1*, *msh2*, *msh3*, *msh6*, *pms1*, and *pms2*. In

another embodiment the gene may be a gene associated with a cancer selected from the

group consisting of a leukemia, a lymphoma, a meningioma, a mixed tumor of a

salivary gland, an adenoma, a carcinoma, an adenocarcinoma, a sarcoma, a

5   dysgerminoma, a retinoblastoma, a Wilms' tumor, a neuroblastoma, a melanoma, and a

mesothelioma.

If an MSH dimer is contacted with a mixture of mismatched duplex

DNA and non-mismatched duplex DNA, the dimer will preferentially associate with

the mismatched duplex DNA. The mismatched duplex DNA is thereby labeled

10   differently than the non-mismatched duplex DNA, and MSH dimer associated with

mismatched duplex DNA may be detected as describe herein or separated from the

non-mismatched duplex DNA. By separating the dimer from non-mismatched duplex

DNA, the mismatched duplex DNA bound to the dimer is separated from the non-

mismatched duplex DNA. Furthermore, mismatched duplex DNA may be dissociated

15   from the dimer after separating it from the non-mismatched duplex DNA.

Methods of detecting an MSH dimer associated with mismatched

duplex DNA include, but are not limited to, electrophoretic gel mobility shift assays,

HPLC and other column and thin layer chromatographic methods, filter binding assays,

immunologic detection methods such as ELISA, tagged antibody, and precipitation

20   assays, centrifugal sedimentation methods, optical affinity sensing, 'footprint' and other

nucleolytic cleavage protection assays, and spectroscopic assays.

In a preferred method of detecting specific binding of the MutS

homolog to the duplex DNA molecule, an optical affinity biosensor system (OABS) is

used to detect specific binding. In an OABS system such as the IAsys™ system

25   (Affinity Sensors, Cambridge, United Kingdom), binding and dissociation events can

be detected as one molecule in solution binds to or dissociates from another molecule

immobilized on a detector surface of the system. Thus, an OABS may be used to

detect specific binding between an MSH dimer and a mismatched duplex DNA in any

of the methods of the invention by immobilizing either the MSH dimer or the

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mismatched duplex DNA on the detector surface of the OABS. Specific binding may be differentiated from non-specific binding by comparing binding of an MSH dimer to a duplex DNA molecule known to comprise a mismatched region and binding of the homolog to a duplex DNA molecule known not to comprise a mismatched region.

5   By way of example, the separation of a mismatched duplex DNA from a population of duplex DNAs may be achieved by binding an MSH dimer to a support, contacting the support with the population of duplex DNAs, and rinsing the support with a separating solution which does not comprise the population of duplex DNAs. If the mismatched duplex DNA has a free end, then the separating solution is preferably

10   substantially free of ATP. In this example, a mismatched duplex DNA in the population of duplex DNAs binds to the MSH dimer and thereby becomes associated with the support. The mismatched duplex DNA is segregated from the other duplex DNAs of the population by rinsing the support with the separating solution, which carries the non-mismatched DNA molecules away from the support. Thus, according

15   to this example, the mismatched duplex DNA is physically separated from the non-mismatched duplex DNAs of the population.

It is not necessary that the just-described method result in separation of the mismatched duplex DNA from the population such that the molecule and the population are contained in different containers at the conclusion of the method. By

20   way of example, it is sufficient in the OABS described herein that a mismatched duplex DNA comprising a region associate with the detector surface of the OABS and that non-mismatched duplex DNAs do not associate with the detector surface of the OABS. Thus, for example, in OABS methods for detection of mismatched duplex DNAs, an MSH dimer may be associated with the detector surface of the OABS, whereby a

25   mismatched duplex DNA binds to the homolog in the presence of ADP and is detected, and whereby a non-mismatched duplex DNA does not bind appreciably to the dimer and is not detected.

Mismatched duplex DNA may be dissociated from an MSH dimer after separating the MSH dimer from a population comprising the mismatched duplex DNA

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and non-mismatched duplex DNAs. The mechanism by which this dissociation may be achieved depends upon whether or not the duplex DNA has a free end.

If the duplex DNA has a free end, then the an MSH dimer may be dissociated from the duplex DNA by contacting the dimer-mismatched duplex DNA

5   complex with a solution having a high salt concentration, with a solution comprising EDTA or another magnesium-chelating agent, or with a releasing solution comprising ATP. Preferably, such a releasing solution comprises at least about 0.3 micromolar ATP, more preferably at least about 3 micromolar, more preferably at least about 30 micromolar ATP, and even more preferably much more than 30 micromolar ATP (e.g.

10   200 micromolar ATP or 500 micromolar ATP). If the mismatched duplex DNA has a free end, then the MSH dimer may be dissociated therefrom simply by contacting the dimer with a solution comprising ATP. The MSH dimer may also be dissociated from the mismatched duplex DNA by contacting the dimer-mismatched duplex DNA complex with a gamma-modified ATP analog.

15   If the mismatched duplex DNA does not have a free end, then an MSH dimer may be dissociated from the duplex DNA by contacting the dimer-mismatched duplex DNA complex with a solution which comprises high salt or EDTA or another magnesium-chelating agent. The dimer will not dissociate from the duplex DNA having no free end in the presence of ATP and magnesium ions (e.g. at least about 10

20   nanomolar Mg^{2+}, preferably at least about 1 micromolar Mg^{2+}, and more preferably at least about 100 micromolar Mg^{2+}. However, if a free end is generated on the mismatched duplex DNA, for example, by cleaving a circular DNA, by removing a blocking group from a blocked end of the DNA, or by cleaving the blocked end of the DNA, then the dimer will dissociate from the duplex DNA in the presence of ATP and

25   magnesium ions. It is understood that there may be some situations in which association of MSH dimers is advantageous (e.g. separating DNA associated with MSH from DNA not associated with MSH). In such situations, taking advantage of the property of MSH dimers to exchange ADP→ATP only when a mismatch is present will permit association of multiple copies of the MSH dimer with the DNA, effectively

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increasing the amount of MSH dimer which can be detected using one or more of the methods described herein. This increase may be particularly important where the detection limit of the assay is relatively low.

Mismatched duplex DNA may be separated from a population of duplex
5 DNAs by contacting the population and an MSH dimer and binding the MSH dimer to a support after contacting it with the population, but prior to separating the non-mismatched duplex DNA from the MSH dimer.

It is understood that if acceleration of ATP displacement of ADP bound to an MSH dimer or acceleration of ATP hydrolysis by MSH dimer not bound to
10 duplex DNA is desired, the MSH dimer may be contacted with a MutL homolog to achieve this acceleration. It is furthermore understood that if the MSH dimer is present in molar excess with respect to the mismatched duplex DNA an average of more than one copy of the MSH dimer may be associated with individual copies of the mismatched duplex DNA if ATP is available to the MSH dimer. The average number
15 of copies of the MSH dimer associated with individual copies of the mismatched duplex DNA may be further increased by contacting the MSH dimer with a MutL homolog. Similarly, the average number of copies of the MSH dimer associated with individual copies of the mismatched duplex DNA may be increased by employing solutions which favor formation of ADP-bound MSH dimer and displacement of ADP
20 bound to mismatch-bound dimer by ATP. Such conditions include, but are not limited to, increasing the concentration ADP in the binding solution, increasing the concentration ATP, magnesium, or both, in the binding solution, and increasing the concentration of the dimer in the binding solution.

The properties of MSH dimers described above can be employed in a
25 variety of useful methods including, but not limited to the following. It is understood that other methods which usefully employ the methods described above may be devised by the ordinarily skilled worker in view of the teachings provided herein.

The invention includes a method of segregating a mismatched duplex DNA from a population of DNA molecules. This method comprises contacting an

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MSH dimer and the population in the presence of a binding solution comprising a nucleotide selected from the group consisting of ADP and ATP. In the presence of this binding solution, the MSH dimer associates with the duplex DNA. After contacting the dimer and the population, the MSH dimer is segregated from the population. The

5   duplex DNA is thereby segregated from the population.

The invention also includes a method of detecting a difference between a sample nucleotide sequence and a reference nucleotide sequence. This method comprises annealing a first DNA strand and a second DNA strand to form a duplex DNA. The first DNA strand has the sample nucleotide sequence, and the second DNA

10   strand has a nucleotide sequence which is complementary to the reference nucleotide sequence. If there is a difference between the sample nucleotide sequence and the reference nucleotide sequence, then the duplex DNA will be a mismatched duplex DNA. After annealing the DNA strands, the duplex DNA and an MSH dimer are contacted in the presence of a binding solution as described herein. If the duplex DNA

15   is a mismatched duplex DNA, then the MSH dimer associates with the duplex DNA. After contacting the duplex DNA and the MSH dimer, association of the MSH dimer with the duplex DNA molecule is detected as described herein. Association of the MSH dimer with the duplex DNA molecule is an indication that there is a difference between the sample nucleotide sequence and the reference nucleotide sequence.

20   The invention further includes a method of determining whether a mammal is predisposed for carcinogenesis. This method comprises annealing a first DNA strand and a second DNA strand to form a duplex DNA. The first DNA strand has the nucleotide sequence of at least a region of an oncogene or a tumor suppressor gene of the mammal, such as one of those described herein. The second DNA strand

25   has a nucleotide sequence which is complementary to the consensus nucleotide sequence of this region. If there is a sequence difference between the first DNA strand and the second DNA strand, then the duplex DNA will be a mismatched duplex DNA. The duplex DNA is contacted with an MSH dimer in the presence of a binding solution as described herein. The MSH dimer associates with the duplex DNA if the duplex

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DNA is a mismatched duplex DNA. After contacting the duplex DNA and the MSH dimer, association of the MSH dimer with the duplex DNA molecule is detected as described herein. Association of the MSH dimer with the duplex DNA molecule is an indication that the mammal is predisposed for carcinogenesis.

5   The invention still further includes a method of fractionating a population of duplex DNAs. This method comprises contacting the population with an MSH dimer in the presence of a binding solution as described herein. The MSH dimer associates with any mismatched duplex DNA in the population. The MSH dimer is segregated from the population, and any mismatched duplex DNA from the population

10   is segregated from the population. The population is thereby fractionated.

The invention also includes a method of selectively amplifying at least one mismatched duplex DNA of a population of duplex DNAs. This method comprises contacting the population with an MSH dimer in the presence of a binding solution as described herein. The MSH dimer associates with the mismatched duplex

15   DNA. The MSH dimer is segregated from the population, and the mismatched duplex DNA is thereby segregated from the population. The mismatched duplex DNA is then amplified.

The invention further includes a method of determining whether the nucleotide sequence of a first copy of a genomic sequence differs from the nucleotide

20   sequence of a second copy of the genomic sequence. This method comprises amplifying a region of each of the first copy and the second copy of the genomic sequence to yield amplified first copies and amplified second copies. The amplified first copies and the amplified second copies are mixed and denatured to form a first mixture. The nucleic acids in the first mixture are annealed to form a second mixture

25   comprising duplex DNAs. If the nucleotide sequence of first copy and the nucleotide sequence of the second copy of the genomic sequence differ, then at least some of the duplex DNAs in the second mixture are mismatched duplex DNAs. The second mixture is contacted with an MSH dimer in the presence of a binding solution as described herein. The MSH dimer associates with any mismatched duplex DNAs that

are present in the second mixture. Association of the MSH dimer with duplex DNA is then detected. Association of the MSH dimer duplex DNA is an indication that the nucleotide sequence of the first copy of the genomic sequence differs from the nucleotide sequence of the second copy of the genomic sequence. The first and second

5   copies of the genomic sequence may be obtained from a single eukaryotic organism or from different eukaryotic individuals of the same or a different species. If the first and second copies of the genomic sequence are obtained from a single individual, one copy may be obtained from each of a pair of the individual's chromosomes. If the first and second copies of the genomic sequence are obtained from different individuals of the

10   same species, then the individuals may, for example, be related, unrelated, or congenic.

The invention yet further includes a composition for segregating a mismatched duplex DNA from a population of duplex DNAs, the composition comprises an MSH dimer bound to a support, and may be used in any of the methods described herein. The composition may be a component of a kit which includes an

15   instructional material which describes a method of the invention wherein the composition is useful. The kit may instead comprise the composition and a binding solution or a releasing solution, as described herein.

The invention also includes a kit for screening a genomic region for a nucleotide sequence which differs from a reference nucleotide sequence. This kit

20   comprises a pair of primers complementary to the ends of the region. The pair of primers is useful for amplifying the region. The kit further includes a DNA strand having the reference nucleotide sequence and at least one MutS homolog. The MutS homolog may be supplied in the form of an MSH dimer. The kit may be used to perform the methods described herein. The kit may further comprise additional

25   components, such as an instructional material which describes use of the kit to perform a method described herein, an assay reagent for detecting binding of a mismatched duplex DNA to the MSH dimer, or a reagent for blocking the ends of duplex DNAs. By way of example, the primers of the kit may be biotinylated and the kit may further comprise an avidin such as streptavidin for blocking the ends of duplex DNA.

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The invention further includes a kit for separating a mismatched duplex DNA from non-mismatched duplex DNAs. This kit comprising at least one MutS homolog, a linker for binding the MutS homolog to a support, and an additional reagent selected from the group consisting of a nucleotide and a releasing solution, as described herein. The releasing solution may, for example, comprise a compound selected from the group consisting of ATP and a gamma-modified ATP analog. The kit may further comprise a reagent for blocking the ends of a duplex DNA, such as biotinylated PCR primers which can be used to amplify the duplex DNA, prior to contacting the biotinylated duplex DNA with an avidin such as streptavidin. Alternately, the kit may comprise a binding solution which is substantially free of ATP, magnesium ions, or both, whereby when a support-bound MSH dimer binds a mismatched duplex DNA, the dimer is not able to bind ATP and magnesium ion, and thus cannot exhibit DNA-associated diffusion and the duplex DNA remains bound to the ADP-bound dimer.

The invention further includes a nonhuman mammal which is nullizygous for both *Msh2* and *p53*. The nonhuman mammal does not express *Msh2* or *p53* and exhibits a phenotype selected from the group consisting of inappropriate fetal apoptosis and a predisposition for carcinogenesis. Preferably, the mammal is a mouse, but other non-human mammals may also be generated using the teaching provided herein.

The invention still further includes a method of making a nonhuman mammal which is nullizygous for both *Msh2* and *p53*. Such a mammal does not express *Msh2* or *p53* and exhibits a phenotype selected from the group consisting of a predisposition for inappropriate fetal apoptosis and a predisposition for carcinogenesis. Such mammals are made by mating a first parent mammal comprising at least one null allele of *Msh2* and at least one null allele of *p53* and a second parent mammal comprising at least one null allele of *Msh2* and at least one null allele of *p53*. The offspring of the two parent mammals inherit the null alleles of these two genes according to normal allelic segregation rules (i.e. generally speaking, most mammals will randomly inherit one of each parent's two alleles of a gene). Thus, the proportion

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of nonhuman mammals which are nullizygous for both *Msh2* and *p53* will depend upon the allelic composition of the parents. Offspring which are nullizygous for both *Msh2* and *p53* do not express *Msh2* or *p53* and exhibit a phenotype selected from the group consisting of inappropriate fetal apoptosis and a predisposition for carcinogenesis.

5   Further details relating to this method are described herein, such as in Example 2.

The invention also includes several screening methods, all of which make use of the properties of the $Msh2^{-/-}p53^{-/-}$ mice described herein.

A standard screening procedure is now described which is useful for determining the tumorigenesis-, apoptosis-, aging-, or fetal development-modulating

10   potential of a compound. While this procedures is described with respect to particular protocols and mice, it will be appreciated that the screening procedure described should not be construed to limit the invention in any way.

$Msh2^{-/-}p53^{-/-}$ mice are generated as described herein or obtained from a producer of such mice. A predetermined amount of the compound is administered to a

15     $Msh2^{-/-}p53^{-/-}$ mouse by any practical means. The method of administration of the compound is not critical. By way of example, the compound may be administered orally, intraperitoneally, intravenously, topically, intramuscularly, or via a pulmonary route.

Following administration of the compound, the $Msh2^{-/-}p53^{-/-}$ mouse,

20   each $Msh2^{-/-}p53^{-/-}$ mouse is observed for about four months. Each mouse is examined approximately daily. Every week, each mouse is weighed, observed for any clinically-relevant symptoms, and the number and extent of tumors are assessed.

To reduce any potential for bias, the study is blinded. A first investigator treats all mice with compound(s) and identifiably marks or cages the

25   transgenic mice, so that the nature of the treatments will not be known to a second investigator, who performs all tumor counts, weighing, and general observations.

If the mice are being used to screen for tumorigenesis-modulating compounds, then after observations are completed, the rate of tumor incidence and the tumor yield are determined for each group of $Msh2^{-/-}p53^{-/-}$ mice to which the

compound was applied. A higher or lower rate of tumor incidence or a higher or lower tumor yield for a group of $Msh2^{-/-}p53^{-/-}$ mice to which the compound was applied, compared with the levels of tumor incidence and tumor yield for a group of $Msh2^{-/-}p53^{-/-}$ mice to which the compound was not applied, is an indication that the

5   compound affects tumorigenesis.

If the mice are being used to screen for apoptosis-modulating compounds or fetal development-modulating compounds, then the mice are preferably administered the compound and observed during fetal development. After observations are completed, the prevalence of inappropriate fetal apoptosis and the fetal survival rate

10   are determined for each group of $Msh2^{-/-}p53^{-/-}$ mouse embryos to which the compound was applied. A higher or lower mouse embryos or a higher or lower fetal survival rate for a group of $Msh2^{-/-}p53^{-/-}$ mouse embryos to which the compound was applied, compared with the mouse embryos and fetal survival rate for a group of $Msh2^{-/-}p53^{-/-}$ mouse embryos to which the compound was not applied, is an indication that

15   the compound affects apoptosis or fetal development.

If the mice are being used to screen for aging-modulating compounds, then after observations are completed, the prevalence of at least one symptom of aging (e.g. graying of hair, other changes in coat color, lethargy, or hair loss) are determined for each group of $Msh2^{-/-}p53^{-/-}$ mice to which the compound was applied. A higher or

20   lower prevalence of a symptom of aging for a group of $Msh2^{-/-}p53^{-/-}$ mice to which the compound was applied, compared with the prevalence of the symptom for a group of $Msh2^{-/-}p53^{-/-}$ mice to which the compound was not applied, is an indication that the compound affects aging.

Preferably, groups of $Msh2^{-/-}p53^{-/-}$ mice or embryos are used, with

25   each mouse in a group being treated identically. Also preferred are studies in which one of at least three different dose levels of the compound are applied to the mice or embryos in each of at least three corresponding groups of transgenic mice. It is preferred, where possible, to demonstrate a statistically significant difference ($P < 0.05$)

between the observed phenotype for the first dose level and the observed phenotype for the third dose level.

A cell line may be made using cells obtained from a $Msh2^{-/-}p53^{-/-}$ mouse of the invention. Methods of making a cell line from a cell of a nonhuman animal are well known in the art.

The invention also includes a method of determining whether a composition interferes with the activity of one of the *p53* gene or one of its expression products and a MutS homolog gene or one of its expression products. According to this method, non-human mammals such as mice are generated which are nullizygous for one of the *p53* gene and the gene encoding the MutS homolog. These nullizygous animals are crossed to generate embryos which are also nullizygous for the same gene. The embryos are contacted with the composition, either *in vitro* or *in utero*, and the effects of contacting the embryos with the composition are observed. Increased mortality among the embryos, particularly among the female embryos, is an indication that the composition is able to interfere with the activity of the other of the *p53* gene or one of its expression products and a MutS homolog gene or one of its expression products. Thus, the ability of a composition to increase female embryonic lethality in mouse embryos which are nullizygous for the *p53* gene is an indication that the composition interferes with the activity of a MutS homolog gene or one of its expression products. Similarly, the ability of a composition to increase female embryonic lethality in mouse embryos which are nullizygous for a MutS homolog gene is an indication that the composition interferes with the activity of the *p53* gene or one of its expression products. Preferably, female embryos are selected and used. Also preferably, female embryonic lethality is observed at about 9.5 days gestation. Methods of generating both nullizygous *p53* animals such as mice and nullizygous *msh* gene animals such as mice have been described in the art.

The invention further includes a composition comprising a human MutS homolog fragment, wherein the fragment comprises a MutS homolog interaction region. The fragment may be a polypeptide having as many as all but one amino acid

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residues of the corresponding MutS homolog. The interaction region may be any of the MutS homolog interaction regions described herein or a MutS homolog interaction region having significant homology thereto. By way of example, a MutS homolog interaction region having significant homology to a MutS homolog interaction region described herein may exhibit at least about 50%, and preferably at least about 70%, 85%, 95%, or 99% homology with a MutS homolog interaction region described herein. Thus, by way of example, the interaction region may be completely or significantly homologous to amino acid residues 378-625 of hMSH2, amino acid residues 875-934 of hMSH2, amino acid residues 126-250 of hMSH3, amino acid residues 1050-1128 of hMSH3, amino acid residues 326-575 of hMSH6, or amino acid residues 1302-1360 of hMSH6.

The composition comprising a human MutS homolog fragment of the invention is useful in a method of inhibiting association of a first human MutS homolog and a second human MutS homolog. This method comprises contacting at least one of the first human MutS homolog and the second human MutS homolog with the human MutS homolog fragment of the invention. Without wishing to be bound by any particular theory of operation, it is believed that the fragment will interact with at least one interaction region of one human MutS homolog, thereby preventing that homolog from associating with the other MutS homolog. Such compounds would have utility for inducing apoptosis in animal cells (e.g. human tumor cells) which harbor one or more mutations in their *p53* genes. Such compounds would also be useful for sensitizing animal cells which harbor one or more mutations in their *p53* genes for further treatment using, for example, DNA-damaging agents.

As described herein in Example 5, cDNA encoding hMSH5 has been discovered, and a protein encoded by that cDNA has also been discovered. hMSH5 may be purified in a manner directly analogous to the methods described herein (e.g. by his-tagging) or by other methods well known in the art. The invention thus includes substantially purified hMSH5 and an isolated nucleic acid encoding hMSH5.

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The invention is now described with reference to the following Examples. These Examples are provided for the purpose of illustration only and the invention should in no way be construed as being limited to these examples, but rather should be construed to encompass any and all variations which become evident as a

5   result of the teaching provided herein.

Example 1

The Human Mismatch Recognition Complex hMSH2:hMSH6

Functions as a Molecular Switch

Adenine nucleotide binding by the human hMSH2:hMSH6 mismatch

10   recognition complex functions as a novel molecular switch. The hMSH2:hMSH6 heterodimer is "ON" (i.e. it associates with mismatched DNA) in the ADP-bound form; and "OFF" (i.e. it is not capable of associating with mismatched DNA with which it is not already associated) in the ATP-bound form. The data presented herein establish that the switch is 'turned OFF' by displacement of complex-bound ADP by ATP. ATP-

15   bound complex is recycled to the ADP-bound form, which is capable of binding to mismatched DNA, by intrinsic ATPase activity of the complex.

The materials and methods used in the experiments presented in this Example are now described.

Overexpression and purification of hMSH2:hMSH6

20   Clones encoding hMSH2 and those encoding hMSH6 have been described (Acharya et al., 1996, Proc. Natl. Acad. Sci. USA 93:13629-13634; Fishel et al., 1993, Cell 75:1027-1038). In the experiments described herein, the clone encoding hMSH6 was modified to further encode six histidine residues at the amino terminus of the hMSH6 protein molecule. hMSH3 can be similarly modified and isolated.

25   hMSH2 and hMSH6 were overexpressed in SF9 insect cells using the pFastBac™ dual expression vector (Gibco BRL, Grand Island, NY) as described in the Bac-to-Bac™ baculovirus expression systems protocol (Gibco BRL, Grand Island, NY). Briefly, SF9 cells suspended in approximately 400 milliliters culture medium were infected using the vector, and were then cultured for 48 hours to achieve a cell

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density of approximately 10^6 SF9 cells per milliliter. The cells contained in 200

milliliter aliquots of SF9 cells were harvested by centrifugation at $200 \times$ g, resuspended

in 10 milliliters of buffer A, and frozen at -80°C. Buffer A comprised 300 millimolar

NaCl, 20 millimolar imidazole, 25 millimolar HEPES buffer adjusted to pH 7.8 using

5   NaOH, 10% (v/v) glycerol, 0.5 millimolar phenylmethylsulfonylfluoride (PMSF), 0.8

microgram per milliliter pepstatin, and 0.8 micrograms per milliliter leupeptin.

Cell extracts were prepared by thawing the cells, passing the cells

through a 25 gauge needle, and then ultracentrifuging the extract at 40,000 rotations per

minute in a Beckman Ti60 rotor for 70 minutes, according to known methods. About

10   100 milliliters of infected cells yielded approximately 2 milligrams of hMSH2:hMSH6

protein complex. All of the following protein purification procedures in this Example

were carried out at 4°C.

The supernatant was applied to a 2 milliliter nickel-NTA Superflow™

column (Qiagen, Chatsworth, CA) at a flow rate of 0.15 milliliters per minute using a

15   Pharmacia FPLC system. The column was washed by passing 35 milliliters of buffer

A through the column. After washing the column, the hMSH2:hMSH6 heterodimer

was eluted by applying 30 milliliters of buffer A comprising a linear gradient of

imidazole to the column and collecting the eluent from the column in fractions,

wherein the concentration of imidizole was varied from 20 millimolar to 200

20   millimolar. The hMSH2:hMSH6 heterodimer eluted in fractions containing

approximately 70 millimolar imidizole.

Fractions from the nickel-NTA column which contained peak amounts

of the heterodimer were loaded at a flow rate of 0.2 milliliters per minute directly onto

a 1 milliliter PBE 94 column (a polybuffer exchange column obtained from Pharmacia,

25   Upsala Sweden) which had been equilibrated with buffer B. Buffer B comprised 300

millimolar NaCl, 25 millimolar HEPES buffer adjusted to pH 7.8 using NaOH, 1

millimolar dithiothreitol (DTT), 0.1 millimolar ethylenediaminetetraacetic acid

(EDTA), 10% (v/v) glycerol, 0.5 millimolar PMSF, 0.8 micrograms per milliliter

pepstatin, and 0.8 micrograms per milliliter leupeptin. The PBE 94 column was

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washed by passing 10 milliliters of buffer B through the column. After washing the
column, the hMSH2/hMSH6 complex was eluted by applying 20 milliliters of buffer B
comprising a linear gradient of NaCl to the column and collecting the eluent from the
column in fractions, wherein the concentration of NaCl was varied from 300 millimolar

5   to 1 molar. The hMSH2:hMSH6 heterodimer eluted from the PBE 94 column in
fractions containing approximately 575 millimolar NaCl.

Fractions collected from the PBE 94 column which contained peak
amounts of the heterodimer were dialyzed twice for two hours against 2 liters of a
solution comprising 100 millimolar NaCl, 25 millimolar HEPES buffer adjusted to pH

10   7.8 using NaOH, 1 millimolar DTT, 0.1 millimolar EDTA, and 20% (v/v) glycerol.
Aliquots of the dialyzed solution containing the heterodimer were frozen using liquid
nitrogen and stored at -80°C for several months without detectable loss of activity.

hMSH2, hMSH6, and bovine serum albumin (BSA) contain nearly
identical percentages (12%, 14%, and 13%, respectively) of arginine and heterocyclic

15   amino acids, the amino acids known to interact with the Coomassie Brilliant Blue stain.
Protein concentration in an aliquot comprising the hMSH2:hMSH6 heterodimer was
determined by subjecting a portion of the aliquot to SDS-PAGE using a 6% (w/v)
acrylamide gel, subjecting a known amount of BSA (Boehringer Mannheim,
Indianapolis, IN) to SDS-PAGE using a 6% (w/v) acrylamide gel, staining the SDS-

20   PAGE gels with Coomassie Brilliant Blue, and comparing the intensities of the protein
bands in the gels to a BSA standard on a Coomassie stained 6% SDS PAGE to
calculate protein concentration. The intensities of stained protein bands were measured
using BioRad Gel Doc and Molecular Analyst™ software. This protein quantitation
method revealed the hMSH2 and hMSH6 proteins to be in near exact equimolar

25   proportion in the heterodimer.

Preparation of 39- and 81-base pair oligonucleotide probes

The sequence of the 39-base pair oligonucleotide used in the
experiments presented in this Example was: 5'-CGGCGAATTC CACCAAGCTT
GATCGCTCGA GGTACCAGG-3' (SEQ ID NO: 1). The homologous 39-base pair

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DNA substrate used in the experiments presented in this Example was made by
annealing the 39-base pair oligonucleotide with an oligonucleotide (SEQ ID NO: 2)
which was completely complementary thereto. The G/T mismatched 39-base pair
DNA substrate used in the experiments presented in this Example was made by
5   annealing the 39-base pair oligonucleotide with an oligonucleotide (SEQ ID NO: 3)
which was completely complementary thereto, except that the oligonucleotide
contained a G residue at the nucleotide position complementary to the T residue at
position 20 (numbered in the direction extending from the 5' end to the 3' end) of the
39-base pair oligonucleotide. SEQ ID NO: 2 and SEQ ID NO: 3 are listed in Figure 8.
10   The nucleotide sequence of the 81-base pair oligonucleotide used in the
experiments described in this Example was: 5'-AAAGCTGGAG CTGAAGCTTA
GCTTAGGATC ATCGAGGATC GAGCTCGGTG CAATTCAGCG GTACCCAATT
CGCCCTATAG T-3' (SEQ ID NO: 4). The homologous 81-base pair DNA substrate
used in the experiments presented in this Example was made by annealing the 81-base
15   pair oligonucleotide with an oligonucleotide (SEQ ID NO: 5) which was completely
complementary thereto. The G/T mismatched 81-base pair DNA substrate used in the
experiments presented in this Example was made by annealing the 81-base pair
oligonucleotide with an oligonucleotide (having the nucleotide sequence listed in SEQ
ID NO: 6) which was completely complementary thereto, except that the
20   oligonucleotide contained a T residue at the nucleotide position complementary to the
G residue at position 41 (numbered in the direction extending from the 5' end to the 3'
end) of the 81-base pair oligonucleotide. SEQ ID NO: 5 and SEQ ID NO: 6 are listed
in Figure 8.
        ^{32}P-end-labeled DNA substrates were prepared by incubating single
25   stranded oligonucleotides in the presence of T4 polynucleotide kinase (Promega Corp.,
Madison, WI) and [^{32}P]γ-ATP (NEN Dupont, Wilmington, DE). Excess label was
separated from the labeled DNA substrates using a Centrisep™ column (Princeton
Separations, Princeton, NJ) per the manufacturer's instructions.

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Labeled DNA substrate was annealed with a single-stranded DNA molecule which was either completely complementary thereto or contained a single G/T mismatch. To anneal the labeled DNA substrate with the single-stranded DNA molecule, the labeled molecule was suspended in a solution comprising a 10-fold

5   excess of the single-stranded DNA molecule, 10 millimolar Tris buffer which had been adjusted to pH 7.5 using HCl, 100 millimolar NaCl, and 1 millimolar EDTA. The suspension was heated to 95°C and then slowly cooled to 55°C and was maintained at this temperature for twelve hours. Single-stranded DNA was removed from the suspension by incubating the suspension with benzoylated naphthoylated DEAE

10   cellulose (BND cellulose, Sigma Chemical Co., St. Louis, MO) for twenty minutes in the presence of a solution comprising 1.5 molar NaCl, 20 millimolar Tris buffer which had been adjusted to pH 7.5 using HCl, and 0.5 millimolar EDTA. BND cellulose was then pelleted by centrifuging the suspension for about five minutes using an Eppendorf bench-top centrifuge. Double-stranded DNA, which remained in the supernatant, was

15   separated from the BND cellulose by filtration and was then precipitated by adding ethanol to the supernatant. The double-stranded labeled DNA substrate was resuspended in a solution comprising 10 millimolar Tris buffer which had been adjusted to pH 7.5 using HCl, 100 millimolar NaCl, and 1 millimolar EDTA. Single-stranded DNA could not be detected in the solution, as assessed by 4% (w/v) native

20   PAGE separation of the nucleotides in the solution. Non-^{32}P-labeled oligonucleotides were prepared using analogous methods.

Gel mobility shift assays

Gel mobility shift assays were performed by incubating a hMSH2:hMSH6 heterodimer and 9 femtomoles of either the ^{32}P-labeled homologous

25   81-base pair DNA substrate or the ^{32}P-labeled G/T-mismatched 81-base pair DNA substrate in a buffer comprising 50 millimolar NaCl, 25 millimolar HEPES buffer which had been adjusted to pH 7.5 using NaOH, 1 millimolar DTT, 0.01 millimolar EDTA, and 15% (v/v) glycerol. The buffer included 10 nanograms per microliter of poly dI-dC (Pharmacia LKB Biotechnology Inc., Piscataway, NJ). Poly dI-dC is an

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alternating nucleic acid polymer which does not interfere with binding of the
hMSH2:hMSH6 heterodimer to DNA. In certain experiments described herein, the
incubation mixture further comprised selected concentrations of nucleotides or non-
labeled DNA. In other experiments described herein, the incubation mixture further

5   comprised 1 millimolar $MgCl_2$ or 5 millimolar EDTA. Except as otherwise described
herein, each incubation mixture had a volume of 20 microliters and was incubated for
fifteen minutes at 37°C and then immediately placed on ice. Each incubation mixture
was applied to a gel comprising 4% (w/v) polyacrylamide (29:1 ratio of
acrylamide:*bis*-acrylamide) 4% (v/v) glycerol, 40 millimolar Tris acetate buffer (pH

10   7.8), and 1 millimolar EDTA. Electrophoresis was performed by applying 200 volts to
the gel for two hours. Following electrophoresis, each gel was dried and quantitated
using a phosphoimaging device obtained from Molecular Dynamics.

Footprint assays

Incubation of the hMSH2:hMSH6 heterodimer with ^{32}P-labeled DNA

15   substrates was performed as described for gel mobility shift assays, except that 18
femtomoles of ^{32}P-labeled DNA substrate was used in each assay. Following
incubation, 80 microliters of a buffer comprising 50 millimolar NaCl, 25 millimolar
HEPES buffer which had been adjusted to pH 7.8 using NaOH, 1 millimolar DTT, 10
nanograms per microliter poly dI-dC, 1.25 millimolar $CaCl_2$, 3.1 millimolar $MgCl_2$,

20   10% (v/v) glycerol, and 33 picograms per microliter DNase (Boehringer Mannheim,
Indianapolis, IN) was added to each incubation mixture. The mixtures were incubated
at 37°C for an additional three minutes, and then 0.7 milliliters of a solution having a
pH of 5.2 and comprising 95% (v/v) ethanol and 180 millimolar sodium acetate was
added to each mixture to halt the DNase reaction and to precipitate the nucleic acids

25   present in the mixture.

DNase-treated nucleic acids were resuspended in 4 microliters of a
solution comprising 80% (v/v) formamide, 10 millimolar NaOH, 1 millimolar EDTA,
and 0.1 % (w/v) bromophenol blue. The suspension was heated at 90°C for five
minutes and was applied to a gel comprising 8% (w/v) polyacrylamide (29:1 ratio of

acrylamide:*bis*-acrylamide), 90 millimolar tris-borate buffer (pH 8), and 2 millimolar

EDTA. Following electrophoresis for 2 hours at 200 volts, each gel was dried and

imaged on a phosphoimaging device. Individual bases of the 81-base pair DNA

substrates were identified by Maxam-Gilbert sequencing reactions performed as

5   described (Ausubel et al., 1994, Current Protocols in Molecular Biology, 8th Ed.,

Janssen, ed., John Wiley & Sons, Inc., Boston).

ATPase assays

ATPase activity was measured in a reaction mixture comprising 20

microliters of Buffer P, 500 micromolar non-labeled ATP (except where indicated),

10   and 16.5 nanomolar $[^{32}P]\gamma$-ATP. Buffer P comprised 40 millimolar HEPES which

had been adjusted to pH 7.8 using NaOH, 75 millimolar NaCl, 10 millimolar $MgCl_2$,

1.75 millimolar DTT, and 0.075 millimolar EDTA, and 15 % (v/v) glycerol. Steady

state reaction measurements were made using 60 nanomolar hMSH2:hMSH6

heterodimer and either 240 nanomolar homoduplex 39-base pair DNA substrate or 240

15   nanomolar G/T mismatched 39-base pair DNA substrate. Reaction mixtures were

incubated at 37°C for thirty minutes, and the reaction was stopped by addition of 400

microliters of a solution comprising 10% (w/v) activated charcoal (Sigma Chemical

Co., St. Louis, MO) and 1 millimolar EDTA. Charcoal was pelleted by centrifuging

the mixture at 10,000 rotations per minute for ten minutes. The ^{32}P content of

20   duplicate 100 microliter aliquots of the supernatant was assessed by liquid scintillation.

Initial velocity measurements were made by incubating the

hMSH2:hMSH6 heterodimer for ten minutes at 25°C in a reaction mixture comprising

one volume Buffer P containing no $MgCl_2$, 200 nanomolar non-labeled ATP, and 16.5

nanomolar $[^{32}P]\gamma$-ATP. To start the reaction, an equal volume of buffer P comprising

25   20 millimolar $MgCl_2$ and 1 millimolar non-labeled ATP was mixed with the reaction

mixture, which raised the $MgCl_2$ and ATP concentrations to 10 millimolar and 500

micromolar, respectively. Aliquots were removed at selected times and

electrophoresed as described herein. A control aliquot was removed and prepared for

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electrophoresis prior to addition of the $MgCl_2$-containing Buffer P to the reaction mixture.

ADP exchange assays

        The ADP-ATP exchange rate was determined in a reaction mixture

5   which comprised Buffer Q, 2.3 micromolar [^{3}H]-ADP, and 60 nanomolar hMSH2:hMSH6 heterodimer. Buffer Q comprised 25 millimolar HEPES which had been adjusted to pH 7.8 using NaOH, 75 millimolar NaCl, 10 millimolar $MgCl_2$, 1 millimolar DTT, and 15% (v/v) glycerol. This reaction mixture was incubated for ten minutes at room temperature. 240 nanomolar G/T-mismatched 39-base pair DNA

10   substrate was added to the reaction mixture, and the incubation was continued for an additional ten minutes. The final volume of the reaction mixture was 10 microliters. The order of addition of DNA and ADP did not affect the kinetic results obtained using this assay. An equal volume Buffer Q comprising 1 millimolar non-labeled ATP was then added to the reaction mixture. Reactions were incubated at 25°C for a selected

15   time and then halted by diluting the reaction mixture with 4 milliliters of an ice-cold stop buffer comprising 25 millimolar HEPES which had been adjusted to pH 7.8 using NaOH, 100 millimolar NaCl, and 10 millimolar $MgCl_2$.

        Each halted reaction mixture was immediately filtered on a HAWP nitrocellulose membrane (Millipore, Bedford, MA) and washed thrice with 4 milliliters

20   of the ice-cold stop buffer. Each filter was air dried and incubated overnight in a standard scintillation cocktail. Radioactivity retained on the filters was quantified using a Beckman scintillation counter. A control reaction mixture was prepared by not adding the Buffer Q comprising 1 millimolar non-labeled ATP to the reaction mixture. The amount of [^{3}H]-ADP retained on the membrane to which the control reaction

25   mixture was applied was considered to correspond to the amount of radioactivity retained when 100% of the complex had [^{3}H]-ADP bound thereto.

Thin Layer Chromatography (TLC) Analysis

        TLC was used to determine the composition of an ATPase reaction mixture which was prepared as described herein in the presence of the G/T-mismatched

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39-base pair DNA substrate, 15 micromolar ATP, and 0.01 micromolar $[^{32}P]\alpha$-ATP and which was permitted to react for twenty minutes at 37°C. TLC was performed as previously described (Fishel et al., 1988, Proc. Natl. Acad. Sci. USA 85:36-40).

The results of the experiments presented in this Example are now

5   described.

Overexpression and purification of the hMSH2-hMSH6 protein complex

hMSH2 and hMSH6 proteins were overexpressed in insect cells using a dual expression baculovirus vector, as assessed by the SDS-PAGE analysis of proteins obtained from cell extract. Co-expression of hMSH2 and hMSH6 proteins resulted in

10   formation of a completely soluble hMSH2:hMSH6 heterodimer. Independent expression of either protein alone resulted in formation of a substantial amount of insoluble protein product. hMSH2 and hMSH6 likely exist together as a highly stable complex *in vivo*, as judged by the results obtained in the experiments described in this Example, the ability of investigators to co-purify these two proteins from human cells

15   (Drummond et al., 1995, Science 268:1909-1912), and the ability of these two proteins to interact *in vitro* (Acharya et al., 1996, Proc. Natl. Acad. Sci. USA 93:13629-13634).

Purification of hMSH2 and hMSH6 from insect cells indicated that a stable heterodimer of the two proteins had been formed. Quantitative densitometry of Coomassie-stained products consistently revealed that the hMSH2 and hMSH6

20   subunits were present in an equimolar ratio, as was observed with the yeast MSH2:MSH6 protein complex (Alani et al., 1997, Mol. Cell Biol. 17: 2436-2447). The purification methodology described herein yielded a protein preparation which was more than 95% homogeneous, which exhibited high MSH2/MSH6 activity, and which appeared to be free of any contaminating nucleic acid or nucleotide.

25   G/T mismatch binding by hMSH2:hMSH6 is a model for mismatch recognition

The hMSH2:hMSH6 heterodimer has been demonstrated herein and by others to bind to the eight possible mismatched nucleotide combinations, as well as to a subset of single nucleotide insertion/deletion mismatches (Acharya et al., 1996, Proc. Natl. Acad. Sci. USA 93:13629-13634; Drummond et al., 1995, Science 268:1909-

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1912; Hughes et al., 1992, J. Biol. Chem. 267:23876-23882). The G/T mismatch was chosen as a model for quantitative analysis of hMSH2:hMSH6 mismatch binding because of its apparently intermediate-to-high recognition specificity, as indicated, for example, by the data presented in Figures 1A-1D.

5   The apparent dissociation constant (K_d) was determined in a simple buffer system comprising neither an adenine nucleotide nor magnesium using the homologous 81-base pair DNA substrate and the G/T-mismatched 81-base pair DNA substrate described herein. Results obtained using both gel shift assays, as depicted in Figure 1A, and DNase footprint assays, as depicted in Figure 1C, indicated that K_d of

10   the hMSH2:hMSH6 heterodimer for G/T mismatches was 20 ± 5 nanomolar. Binding of non-mismatched DNA to the heterodimer was not saturable, even at homoduplex concentrations greater than 400 nanomolar.

          The binding of the hMSH2:hMSH6 heterodimer to a G/T mismatch is at least ten times more efficient than binding of hMSH2 alone to the G/T mismatch

15   (Fishel et al., 1994, Science 266:1403-1405; Fishel et al., 1994, Cancer Res. 54:5539-5542; Mello et al., 1996, Chemistry & Biology 3:579-589). This observation indicates that formation of the hMSH2:hMSH6 heterodimer enhances both the affinity and the specificity of hMSH2-binding to mismatched DNA (Acharya et al., 1996, Proc. Natl. Acad. Sci. USA 93:13629-13634).

20   Gel mobility shift assays performed using the G/T-mismatched 39-base pair DNA substrate described herein or using the G/T-mismatched 81-base pair DNA substrate and a buffer comprising 2 millimolar $MgCl_2$ yielded results similar to those shown in Figure 1A. The hMSH2:hMSH6 heterodimer appears to bind G/T mismatched DNA in multiple forms which are differentiable by gel mobility shift

25   assay.

          DNase footprint analysis of hMSH2:hMSH6 heterodimer binding to the G/T-mismatched 81-base pair DNA substrate indicated that the complex asymmetrically protects about 25 nucleotides on both strands of the substrate. As shown in Figure 1C, there appeared to be two domains protected by the complex from

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cleavage by DNase. One domain appeared to be centered on the G/T mismatch in the

substrate. The other domain was adjacent the domain centered on the G/T mismatch

and was separated from that domain by a single DNase-sensitive nucleotide. These

data are qualitatively similar to those observed in similar experiments using the *E. coli*

5   and *T. aquaticus* MutS proteins (Su et al., 1986, Proc. Natl. Acad. Sci., USA 83:5057-

5061; Su et al., 1988, J. Biol. Chem. 263:6829-6835; Biswas et al., 1997, J. Biol.

Chem. 272: 13355-13364).

Although a shifted complex could be detected by gel mobility shift

assay using homoduplex DNA, no specific DNase footprint could be identified, as

10   indicated by the data presented in Figure 1D. Lack of saturatability and lack of a

specific footprint are consistent with the ability of the hMSH2:hMSH6 heterodimer to

weakly and non-specifically associate with homoduplex DNA.

Shifted complexes formed between the heterodimer and homoduplex

DNA and those formed between the heterodimer and G/T-mismatched DNA migrated

15   differently in gel mobility shift assays, as shown in Figures 1A and 1B. Homoduplex

DNA-bound heterodimer (designated 'NS' for 'non-specific' in Figure 1B) migrated

more slowly than G/T-mismatched DNA-bound heterodimer (designated 'S' for

'specific' in Figure 1A). These results suggest that homoduplex DNA-bound

heterodimer adopts a different conformation than mismatched DNA-bound

20   heterodimer. Alternatively, there may have been a greater quantity of the heterodimer

bound to homoduplex DNA than to mismatched DNA.

When the homoduplex 39-base pair DNA substrate described herein was

contacted with the heterodimer, no NS product was observed in the gel mobility shift

assay. The DNA length dependence of NS product formation may result if a minimum

25   number of base pairs were necessary to assume an alternative DNA and/or hMSH2- or

hMSH6-protein conformation or to bind multiple hMSH2:hMSH6 heterodimers.

These results demonstrate the high specificity of heterodimer binding to

the G/T-mismatched 81-base pair DNA substrate. The binding was found to be

quantitatively similar by both gel mobility shift and footprint analysis. In addition, a

low level non-specific binding to duplex DNA was observed and found to be easily

distinguished via its altered mobility using gel mobility shift analysis.

The hMSH2:hMSH6 heterodimer converts ATP to ADP in the presence of mismatched DNA

5   Both bacterial and yeast MutS homologs have been shown to possess

intrinsic low-level ATPase activity (Alani et al., 1997, Mol. Cell Biol. 17: 2436-2447;

Chi et al., 1994, J. Biol. Chem. 269: 29993-29997; Chi et al., 1994, J. Biol. Chem.

269:29984-29992; Habe et al., 1988, J. Bacteriol. 170:197-202). There are conflicting

reports regarding the capacity of mismatched heteroduplex and/or homoduplex DNA to

10   stimulate this intrinsic ATPase activity (Alani et al., 1997, Mol. Cell Biol. 17: 2436-

2447; Chi et al., 1994, J. Biol. Chem. 269: 29993-29997; Chi et al., 1994, J. Biol.

Chem. 269:29984-29992).

It was demonstrated in the experiments described in this Example that

the hMSH2:hMSH6 heterodimer possesses intrinsic DNA-dependent ATPase activity

15   that is dependent upon the presence of magnesium as a cofactor. Saturation of the

ATPase activity by hMSH2:hMSH6 heterodimer which was observed at protein

concentrations above 0.6 micromolar was likely the result of a limiting amount of

DNA, which was use at a fixed concentration of 240 nanomolar in the assay.

Thin layer chromatography revealed that hMSH2:hMSH6 heterodimer

20   ATPase activity uniformly converts ATP to ADP and inorganic phosphate. Using

Lineweaver-Burk analysis and Eadie-Hofstee analysis, it was determined that

hMSH2:hMSH6 heterodimer ATPase is most active in the presence of a G/T mismatch.

The value of k_{cat} using ATP and G/T-mismatched DNA as substrates was about 26

minute^{-1}. The value of K_m using ATP and G/T-mismatched DNA as substrates was

25   about 46 micromolar. hMSH2:hMSH6 heterodimer ATPase is substantially less active

in the presence of homoduplex DNA. The value of k_{cat} using ATP and G/C-

mismatched DNA as substrates was about 7.4 minute^{-1}. The value of K_m using ATP

and G/C-mismatched DNA as substrates was about 23 micromolar. hMSH2:hMSH6

heterodimer ATPase is substantially inactive in the absence of DNA. The value of k_{cat}

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using ATP alone as a substrate was about 0.9 minute^{-1}. The value of K_m using ATP alone as a substrate was about 10 micromolar.

ATPase activity stimulation was the same regardless of whether the homoduplex DNA had a length of 39 base pairs, 81 base pairs or 2,900 base pairs, and was also the same regardless of whether the mismatched DNA had a length of 39 base pairs or 81 base pairs. These results indicated that hMSH2:hMSH6 heterodimer ATPase activity is not dependent upon DNA length.

It was observed that k_{cat} using ATP alone as a substrate was lower than k_{cat} using ATP and homoduplex DNA as a substrate and this value was lower than k_{cat} using ATP and mismatched DNA as substrates. However, K_m for ATP in the absence of DNA was lower than K_m for ATP in the presence of homoduplex DNA, and this value was lower than K_m for ATP in the presence of mismatched DNA. These observations indicated that although the rate of hydrolysis is increased in the presence of a mismatch, the affinity for ATP is decreased. These results are qualitatively similar to the phenomenon of uncompetitive inhibition which may be ascribed to the presence of independent and separate binding sites as well as a ping-pong binding mechanism (Dixon et al., 1979, Enzymes, 3rd Ed., Academic Press, New York).

Single-stranded DNA (ssDNA) was determined to be the most potent stimulator of hMSH2:hMSH6 heterodimer ATPase activity. Thus, the conflicting reports in the prior art regarding ATPase activities of related MutS homologues may have resulted from contamination by ssDNA leached from columns used to purify the homologues and/or by non-annealed ssDNA that remained following preparation of oligonucleotide substrates.

hMSH2:hMSH6 heterodimer mismatch binding is abolished in the presence of ATP in the absence of hydrolysis of ATP

Both bacterial and eukaryotic MutS homologs have been reported to fail to form a specific complex with a mismatched oligonucleotide in the presence of ATP (Drummond et al., 1995, Science 268:1909-1912; Haber et al., 1991, EMBO. J. 10:2707-2715; Alani et al., 1997, Mol. Cell Biol. 17: 2436-2447; Grilley et al., 1989, J.

Biol. Chem. 264:1000-1004). Before the present invention, it was believed that ATP

hydrolysis catalyzed by MutS protein drove translocation of the protein along a duplex

DNA strand, causing dissociation of the protein from any mismatch with which it

might be associated (Grilley et al., 1989, J. Biol. Chem. 264:1000-1004; Modrich,

5   1989, J. Biol. Chem. 264:6597-6600; Modrich, 1991, Annu. Rev. Genet. 25:229-253;

Modrich et al., 1996, Annu. Rev. Biochem. 65:101-133; Allen et al., 1997, EMBO J.

16:4467-4476). The suggestion that ATP hydrolysis was required for the mismatch

release was based on the observation by others that adenylyl-imidodiphosphate (AMP-

PNP), a non-hydrolyzable analog of ATP, does not alter mismatch binding (Alani et al.,

10   1997, Mol. Cell. Biol. 17: 2436-2447; Drummond et al., 1995, Science 268:1909-

1912).

        The experiments described in this Example establish that the

hMSH2:hMSH6 heterodimer is released from a G/T-mismatched DNA substrate in the

presence of ATP, as indicated in Figures 2A and D. The value of IC_{50} (the

15   concentration of ATP required to cause release of 50% of a population of heterodimers

from a G/T-mismatched DNA substrate) was determined to be approximately 3

micromolar. Adenosine-5'-O-3-thiotriphosphate (ATP-γ-S), a poorly-hydrolyzable

ATP analog (Sekimizu et al., 1987, Cell 50:259-265; Yu et al., 1992, J. Mol. Biol.

225:193-216), caused a similar release of the hMSH2:hMSH6 heterodimer from a G/T-

20   mismatched DNA substrate, the value of IC_{50} for ATP-γ-S being 3 micromolar, as

indicated in Figures 2B and 2D. Addition of ADP to the mismatch binding reaction

mixture resulted increased binding affinity of the heterodimer for the G/T-mismatched

DNA substrate, as indicated in Figures 2C and 2D.

        The results presented in this Example demonstrate that release of the

25   hMSH2:hMSH6 heterodimer from a G/T-mismatched DNA substrate with which it is

associated is not dependent upon ATP hydrolysis. This conclusion follows from the

observations that release of the complex occurs in the absence of exogenous

magnesium and that release of the complex from the substrate is effected by the

presence of ATP-γ-S regardless of the presence or absence of magnesium. The

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presence of magnesium is absolutely required for hMSH2:hMSH6 heterodimer-dependent ATP hydrolysis. Furthermore, NS binding of hMSH2 to homoduplex DNA is insensitive to the addition of exogenous ATP. Thus, the presence of ATP affects only the ability of the hMSH2:hMSH6 heterodimer to bind to mismatched DNA

5   substrates. Binding of the heterodimer to homoduplex DNA is not affected by ATP.

The presence of 2'-deoxy adenosine triphosphate (dATP) to the mismatch binding reaction mixture caused release of a G/T-mismatched DNA substrate from the hMSH2:hMSH6 heterodimer, similarly to the release caused by the presence of ATP or ATP-γ-S in the mixture, as illustrated in Figure 3. No other nucleotide was

10   found to stimulate the release of the G/T-mismatched DNA substrate from the heterodimer.

Neither of two other non-hydrolyzable analogs of ATP, namely AMP-PNP and adenyl-(β-, γ-methylene)-diphosphonate (AMP-PCP), caused release of the heterodimer from the substrate. Equilibrium competition between each of these two

15   analogs and ATP suggested that they bind to the heterodimer and caused effects similar to those caused by ADP. Failure of AMP-PNP and AMP-PCP to stimulate release of mismatched DNA from the heterodimer demonstrated that the interaction between the β-γ bridging oxygen atom of ATP and either the heterodimer or the mismatched DNA substrate bound to the heterodimer are for release of the substrate from the heterodimer.

20   Enzyme-nucleotide triphosphate complexes in which the β, γ oxygen atom interacts with either the enzyme or its substrate are not unknown. For example, the Ras GTPase binds GTP, and donation of a hydrogen bond to the β-γ bridging oxygen of GTP is thought to contribute to catalysis by the enzyme (Maegley et al., 1996, Proc. Natl. Acad. Sci. USA 93:8160-8166).

25   The results presented in this example demonstrate that the hMSH2:hMSH6 heterodimer binds to a mismatched DNA substrate in the presence of ADP, and that the substrate is released from the heterodimer in the presence of ATP or dATP. Because ATP-induced release of the substrate from the heterodimer does not require magnesium and is similarly induced by ATP-γ-S, ATP hydrolysis is not

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implicated in substrate release. As increasing amounts of ATP or ATP-γ-S were added
to the mismatch binding reaction mixture, approximately 15% of S-shifted material
gradually became re-associated with the DNA in the form of a NS-shifted heterodimer,
as indicated in Figures 2A and 2B. This fraction was consistent with the amount of NS

5   binding observed for homoduplex DNA at this concentration of the heterodimer, as
indicated in Figure 2B. These results indicated that hMSH2:hMSH6 heterodimers
which dissociated from mismatched substrate could re-associate with either the duplex
arms or the ends of the substrate.

ATP hydrolysis catalyzed by the hMSH2:hMSH6 heterodimer results in recovery of

10   mismatch binding activity of the heterodimer

To determine the role of ATP hydrolysis in mismatch recognition, ATP
or ATP-γ-S was introduced into a mismatch binding reaction mixture in the absence of
magnesium. As illustrated in Figures 2A, 2B, 2D, and 3, introduction of either
compound resulted in release of the hMSH2:hMSH6 heterodimer from the mismatched

15   DNA substrate in the absence of hydrolysis of the compound. In experiments
presented in Figure 4A, magnesium was added to each reaction mixture, which was
maintained at 37°C, and the G/T mismatch binding activity of hMSH2:hMSH6
heterodimer was followed over time, with time zero corresponding to the time at which
magnesium was added. In the reaction mixture comprising ATP, mismatched DNA

20   substrate binding activity of the heterodimer was initially low, nearly 70% of this
activity was recovered after ten minutes of incubation at 37°C, and more than 95% of
the activity was recovered fifty minutes after magnesium addition. Substantially less
(about 22%) of mismatched DNA substrate binding activity was recovered in the
reaction mixture to which ATP-γ-S was added. These results demonstrated that

25   efficient hydrolysis by the heterodimer is essential for recovery of the heterodimer's
mismatch binding activity. Substitution of ATP with dATP produced quantitatively
similar recovery of mismatch binding activity (i.e. >95% recovery) following
incubation at 37°C. Taken together, these results demonstrated that the intrinsic
ATPase activity associated with the human hMSH2:hMSH6 heterodimer is required for

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recovery from mismatch-release induced by binding to and/or exchange with, ATP or dATP.

Complete recovery of mismatched DNA substrate binding activity of the hMSH2:hMSH6 heterodimer, which activity was abolished by exposing the

5   heterodimer to ATP, was achieved by increasing the ratio of the concentration of ADP to the ratio of ATP in the solution in which the heterodimer was suspended, as indicated in Figure 4B In this competition experiment, mismatch binding reaction mixtures comprised 0.2 millimolar ATP, 1 millimolar $MgCl_2$, and a selected concentration of ADP from 0 to 3.2 millimolar. It was determined that a 2- to 3-fold

10   excess of ADP to ATP resulted in reversal of approximately half of the release of substrate by the heterodimer caused by the presence of ATP. Approximately complete reversal of substrate release caused by the presence of ATP was achieved by providing a 16-fold excess of ADP to the mixture. A qualitatively similar, though functionally opposite, result was obtained when the competition was performed by including a fixed

15   concentration of ADP in the reaction mixture and adding various concentrations of ATP. Thus, ADP and ATP are nearly equivalent in their ability to associate with the hMSH2:hMSH6 heterodimer, but the two nucleotides elicited opposite functional effects on mismatch binding. ATP caused release of substrate bound to the heterodimer, and ADP induced binding of the substrate to the heterodimer. Therefore,

20   ADP is responsible for mismatch binding recovery.

Taken together, these observations support the conclusion that the hMSH2:hMSH6 heterodimer functions as a molecular switch, wherein the ATP- (or dATP-) bound heterodimer is "OFF" (i.e. unable to associate with a mismatched DNA substrate with which it is not already associated) and the ADP-bound heterodimer is

25   "ON" (i.e. able to associate with a mismatched DNA substrate with which it is not already associated). A model of the role of the hMSH2:hMSH6 heterodimer is illustrated in Figure 7.

ATP hydrolysis and ADP-ATP exchange determine mismatch binding functions of the
hMSH2:hMSH6 heterodimer

Steady-state analysis of an enzyme having ATPase activity reflects the
rate-limiting step of the reaction, which can be either γ-phosphate hydrolysis or
5   adenine nucleotide exchange. To understand the mechanism of the ATPase activity
exhibited by the hMSH2:hMSH6 heterodimer and to further define the rate-limiting
steps, both hydrolysis and nucleotide exchange steps were directly examined.

Initial rate (i.e. single-turnover) analysis of an enzyme which exhibits
ATPase activity involves direct examination of the rate of γ-phosphate hydrolysis, and
10   was performed using a method which is similar to that used for the examination of
regulators of G-protein signaling (RGS; Dohlman et al., 1997, J. Biol. Chem.
272:3871-3874). In these experiments, 0.2 micromolar [^{32}P]γ-ATP was contacted
with hMSH2:hMSH6 heterodimer in the absence of magnesium, yielding a heterodimer
having a [^{32}P]γ-ATP molecule bound thereto. At a selected time, magnesium and an
15   excess of non-labeled ATP were added to the reaction mixture, and the rate of a single-
round of γ-phosphate hydrolysis was assessed. Subsequent rounds of hydrolysis were
undetectable because the ATP hydrolyzed during those rounds was not labeled.
Because the calculated K_{cat} for ATP at 37°C was in excess of 20 minute^{-1}, and
because this rate was above the limit of detection of this methodology, these initial rate
20   experiments were performed at 20°C. It was determined that the hMSH2:hMSH6
heterodimer rapidly hydrolyzed ATP in either the presence or the absence of DNA.
These results indicated that γ-phosphate hydrolysis was not the rate limiting step in the
steady-state ATP hydrolysis by the heterodimer.

The extent of ATP hydrolysis which could be detected was equivalent to
25   the total number of hMSH2:hMSH6 heterodimers which could be bound to ^{32}P-
labeled ATP prior to the addition of magnesium. The maximal extent of detectable
ATP hydrolysis was determined to depend on the amount of the G/T-mismatched DNA
substrate present in the reaction mixture during binding of labeled ATP to the
heterodimer, as indicated in Figures 5A and 5B. When the concentration of the G/T-

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mismatched DNA substrate in to the reaction mixture exceeded the apparent K_d for G/T-mismatched DNA substrate (i.e. about 20 nanomolar), the maximal extent of ATP hydrolysis decreased, as indicated in Figure 5B. This observation indicated that binding of the hMSH2:hMSH6 heterodimer to a mismatched DNA molecule prior to binding of ATP to the heterodimer inhibits binding of ATP to the mismatched DNA-bound heterodimer. This observation is consistent with the pseudo-uncompetitive behavior deduced in the steady-state ATPase activity experiments described herein (Dixon et al., 1979, Enzymes, 3rd Ed., Academic Press, New York).

Adenine nucleotide exchange was assessed using a method similar to that used for guanine nucleotide exchange experiments involving G proteins. In these studies, [^{3}H]-ADP was contacted with hMSH2:hMSH6 heterodimer in the presence of magnesium, yielding [^{3}H]-ADP-bound heterodimer. At a selected time, an excess of non-labeled ATP was added to the reaction mixture, and the amount of ADP that remained bound to the heterodimer was assessed at selected times.

In the absence of DNA, incomplete ADP nucleotide exchange was observed during a 15 minute reaction period. The half-life of the ADP-bound heterodimer was greater than eight hundred seconds. These results clearly suggest that in the absence of DNA, replacement of ADP by ATP is the rate limiting step for the hMSH2:hMSH6 heterodimer ATPase activity.

In the presence of G/T-mismatched DNA substrate, nucleotide exchange was significantly more rapid, the half-life of the ADP-bound heterodimer being less than two seconds. Thus, it was demonstrated that binding of the heterodimer to a G/T-mismatched DNA substrate stimulated replacement of the labeled ADP molecule originally bound to the heterodimer by a non-labeled ATP molecule.

Taken together with the results obtained from the single turnover hydrolysis experiments described herein, these observations indicated that in the absence of mismatched DNA, the hMSH2:hMSH6 heterodimer is capable of a single ATP hydrolysis reaction that yields an ADP-bound heterodimer. While in the ADP-bound form, the heterodimer does not exchange ADP for ATP until the heterodimer

binds to a DNA mismatch. By binding to a mismatch, the ADP-bound heterodimer

becomes competent to exchange ADP for ATP. Exchange of ADP for ATP causes

release of the heterodimer from the mismatch. ATP-bound heterodimer, when no

longer bound to mismatched DNA, is capable of catalyzing ATP hydrolysis, yielding

5   ADP-bound heterodimer, which is competent to bind to a DNA mismatch. These

results indicate that the hMSH2:hMSH6 heterodimer is a molecular switch controlled

by the phosphorylation state of the adenine nucleotide bound thereto.

Release of the hMSH2:hMSH6 heterodimer from a G/T-mismatched DNA substrate

may occur by dissociation

10   Prior art models of mismatch recognition by MutS homologs implicated

ATP-dependent translocation and/or treadmilling along DNA as a mechanism for

association and dissociation of the homolog with a DNA mismatch (Modrich, 1989, J.

Biol. Chem. 264:6597-6600; Modrich, 1991, Annu. Rev. Genet. 25:229-253; Modrich

et al., 1996, Annu. Rev. Biochem. 65:101-133; Allen et al., 1997, EMBO J. 16:4467-

15   4476). Common to all of these prior art models is a postulated time-dependent

unidimensional homolog displacement mechanism which occurs whether the homolog

is bound to duplex DNA or mismatched DNA. In contrast, a simple dissociation

mechanism would exhibit rapid and two-dimensional displacement of the homolog

from duplex DNA or mismatched DNA.

20   The ability to distinguish NS and S electrophoretic bands corresponding

to the homologous 81-base pair DNA substrate-bound hMSH2:hMSH6 heterodimer

and the G/T-mismatched 81-base pair DNA substrate-bound heterodimer, as illustrated

in Figure 2A, provided an opportunity to examine the dissociation mechanism of the

heterodimer from the G/T-mismatched DNA substrate, as well as from homoduplex

25   DNA. In these experiments, the G/T-mismatched DNA substrate was bound to the

heterodimer, and an excess of an unlabeled competitor DNA or an excess of ATP, or

both, was added to the mixture. If a tracking or sliding mechanism of the prior art were

operable for heterodimer dissociation, it would be expected that a time-dependent loss

of the S shifted electrophoretic band of G/T-mismatched DNA substrate-bound

complex would be observed, and that a coincident gain of the NS electrophoretic band would be observed. If a simple dissociation mechanism were operable for heterodimer dissociation, it would be expected that loss of the S shifted band would be observed without any coincident increase in the intensity of the NS shifted band because the vast

5   excess of unlabeled homoduplex DNA would preclude secondary reassociation of the complex with the arms or ends of the labeled G/T-mismatched DNA substrate. One potential complication would be if the amount of time required for heterodimer enables diffusion of the dimer to a different position on the DNA substrate were nearly the same as the time which would be required for simple dissociation.

10   Three experiments were performed to determine the mechanism of hMSH2/hMSH6 protein complex dissociation from a labeled 81-base pair G/T-mismatched DNA substrate. The results of these experiments are illustrated in Figure 6.

            In the first experiment, the stability of G/T-mismatched DNA substrate-

15   bound hMSH2/hMSH6 complex was assessed by exposing the mismatched substrate-bound complex to a 400-fold excess of non-labeled homoduplex DNA and observing the intensities of S shifted and NS shifted electrophoretic bands at selected times, as illustrated in Figure 6C. Examination of the gel depicted in Figure 6C indicated that the S-shifted electrophoretic band, and thus the amount of the G/T-mismatched DNA

20   substrate-bound hMSH2:hMSH6 heterodimer in the reaction mixture, was not reduced significantly over the ten minute incubation period. Thus, the half-life of the G/T-mismatched DNA substrate-bound hMSH2:hMSH6 heterodimer was much greater than ten minutes, meaning that the mismatched substrate-bound complex is stable in the presence of a vast excess of homoduplex DNA.

25   In the second experiment, the stability of G/T-mismatched DNA substrate-bound hMSH2:hMSH6 heterodimer was assessed by exposing the mismatched substrate-bound heterodimer to ATP and observing the intensities of S shifted and NS shifted electrophoretic bands at selected times, as illustrated in Figure 6A. A gradual decrease in the intensity of the S shifted electrophoretic band was

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observed, the band having a half life of about twenty seconds. Concurrently with the decrease in the intensity of the S shifted electrophoretic band, a gradual but not quantitative increase in the intensity of the NS-shifted electrophoretic band was observed. This observation indicated that ATP induced a time-dependent reduction of

5   specific binding of the hMSH2:hMSH6 heterodimer to the mismatched DNA substrate and that at least a portion of the heterodimer reassociated with the mismatched DNA substrate in a non-specific manner. However, this experiment did not distinguish between the tracking/sliding or simple dissociation and reassociation mechanisms.

In order to attempt to distinguish between translocation and simple

10   dissociation and reassociation, a third experiment was performed. In this experiment, the stability of G/T-mismatched DNA substrate-bound hMSH2:hMSH6 heterodimer was assessed by exposing the mismatched substrate-bound heterodimer to both ATP and a 400-fold excess of non-labeled homoduplex DNA and observing the intensities of S shifted and NS shifted electrophoretic bands at selected times (Figure 6B). As in

15   the second experiment, a gradual decrease in the intensity of the S shifted electrophoretic band was observed, the half-life of the band again being about twenty seconds. This observation was consistent with ATP induction of dissociation of the heterodimer from the mismatched DNA substrate. However, under these conditions, no increase in the intensity of the NS electrophoretic band was observed. Together,

20   these observations suggest that in the presence of excess non-labeled homoduplex DNA, the dissociation of the heterodimer from mismatched DNA might not proceed through the product corresponding to the NS electrophoretic band, but instead may be instantaneous and irreversible.

When excess non-labeled homoduplex DNA was added to the

25   homologous 81-base pair DNA substrate, the NS electrophoretic band associated with the product formed by contacting the heterodimer with DNA substrate, as indicated in Figure 1B, for example, could be detected, as indicated in Figure 6D. This observation indicated that, even at 4°C, the product corresponding to the NS band was exceedingly unstable and that the level of hMSH2:hMSH6 heterodimer which remained associated

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with the DNA substrate was less than the lower limit of accurate quantitation using gel shift analysis.

The hMSH2:hMSH6 heterodimer acts as a molecular switch in mismatch recognition

The discovery that the hMSH2:hMSH6 heterodimer is a novel

5   molecular switch which is activatable by ADP was made by reconciling numerous observations described herein. These observations are summarized as follows. ADP and ATP have opposing effects on the role of the hMSH2:hMSH6 heterodimer in mismatched DNA binding. Dissociation of mismatched DNA from the hMSH2:hMSH6 heterodimer is not dependent upon ATP hydrolysis. Hydrolysis of

10   ATP by the hMSH2:hMSH6 heterodimer results in recovery of the ability of the heterodimer to associate with mismatched duplex DNA. γ-Phosphate hydrolysis is not the rate limiting step of ATPase activity catalyzed by the of the heterodimer. Displacement of ADP by ATP is the rate limiting step of ATPase activity catalyzed by the hMSH2:hMSH6 heterodimer. Displacement of ADP from the of the heterodimer

15   by ATP is accelerated in the presence of mismatched duplex DNA, but hydrolysis of the γ-phosphate bond is not accelerated. ATP-dependent release of mismatched DNA from the hMSH2:hMSH6 heterodimer occurs rapidly, possibly by simple dissociation or by rapid ATP-hydrolysis-independent diffusion to a free end of the DNA. These observations indicate that γ-phosphate hydrolysis and displacement of ADP by ATP

20   determine whether the hMSH2:hMSH6 heterodimer binds to or is released from mismatched duplex DNA, as illustrated in Figure 7. Recognition of the hMSH2:hMSH6 heterodimer as a molecular switch supports the conclusion that it is a trigger for determining the timing of subsequent excision repair-related events.

Implications for mismatch repair

25   The number of hMSH2:hMSH6 heterodimers in the nucleus of a proliferating cell has been estimated to exceed one thousand (Drummond et al., 1995, Science 268:1909-1912; Wilson et al., 1995, Cancer Res. 55:5146-5150; Meyers et al., 1997, Cancer Res. 57:206-208). The calculated K_d of the heterodimer for mismatched DNA (i.e. about 20 nanomolar) implies that a single mismatched nucleotide in a human

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cell is likely to be efficiently recognized and bound with high affinity by an
hMSH2:hMSH6 heterodimer. In the presence of ATP, this high affinity binding is
nearly irreversible. Thus, dissociating the heterodimer from mismatched DNA in order
to allow a subsequent excision repair event to proceed may be more difficult than

5   binding the heterodimer to the mismatch.

Generality of MutS function

The studies described in this Example, which involved the human
mismatch binding reaction catalyzed by the hMSH2:hMSH6 heterodimer, are
consistent with genetic studies performed in both bacteria and yeast. In those studies,

10   mutation of the adenine nucleotide binding and hydrolysis domain(s) resulted in a
dominant mutator phenotype (Haber et al., 1991, EMBO. J. 10:2707-2715; Wu et al.,
1994, J. Bacteriol. 176:5393-5400; Alani et al., 1997, Mol. Cell. Biol. 17: 2436-2447).
Those studies, combined with the studies described in this Example, indicate that there
may be two opposing functional alterations of MutS homologs that can cause such a

15   dominant mutator phenotype. First, alteration of the ability of the homolog to bind
and/or exchange ADP for ATP can cause a dominant mutator phenotype. Second,
alteration of the ability of the homolog to hydrolyze ATP can similarly cause such a
phenotype. Inability of the homolog to bind to ADP or to exchange ADP for ATP
would result in a permanently mismatched DNA-bound form of the MutS homolog.

20   This form of the homolog would exclude the repair machinery from the mismatch site.
Inability of the MutS homolog to hydrolyze ATP would result in a form of the
homolog that would be unable to bind to mismatched DNA and which, therefore,
would be unable to recruit the cellular mismatch repair proteins and factors to the site
of the mismatch. Each these conditions would cause an increased mutation rate in the

25   organism containing the homolog, as a consequence of the organism's depressed ability
to repair mismatched DNA (Wu et al., 1994, J. Bacteriol. 176:5393-5400).

Preliminary studies performed using the methods described herein and
using purified *Escherichia coli* MutS protein suggest that *E. coli* MutS also functions
as a molecular switch, albeit with a more stringent requirement for mismatch-induced

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nucleotide exchange. Therefore, the properties of the MutS homologs hMSH2 and hMSH6, as described herein appear to be properties of all MutS homologs, including, but not limited to, *E. coli* MutS, and the human MutS homologs hMSH2, hMSH3, and hMSH6.

5   Similarity of the hMSH2:hMSH6 heterodimer to G-protein switches

The hMSH2:hMSH6 molecular switch is, in some respects, similar to G-protein switches which have been described (Bokoch et al., 1993, FASEB J. 7:750-759). G-proteins are known to trigger translocation events associated with protein synthesis (Laalami et al., 1996, Biochimie 78:577-589; Parmeggiani et al., 1981, Mol.

10   Cell. Biochem. 35:129-158), cascade events associated with cell signaling (Mederna et al., 1993, Crit. Rev. Oncol. 4:615-661; Wiesmuller et al., 1994, Cell Signal. 6:247-267) and physiological responses to ligand-binding by membrane receptors (Spiegel, 1987, Mol. Cell. Endocrinol. 49:1-16). Many G-proteins are associated with regulators that stimulate both the GTPase activity of the G-protein (Tocque et al., 1997, Cell Signal.

15   9:153-158) and the exchange of G-protein-bound GDP for GTP (Dohlman et al., 1997, J. Biol. Chem. 2 72:3871-3874; Quilliam et al., 1995, Bioessays 17:395-404). In fact, the Ras G-protein was determined to be unable to catalyze GTP hydrolysis because it is unable to exchange GDP for GTP. The discovery of a GTPase activating protein (GAP) that stimulated GTP γ-phosphate hydrolysis, and a guanine nucleotide exchange

20   factor (GNEF) that stimulated the exchange of GDP for GTP, provided a model for regulation of the Ras G-protein switch (Tocque et al., 1997, Cell Signal. 9:153-158; Dohlman et al., 1997, J. Biol. Chem. 2 72:3871-3874).

It has therefore been discovered that protein regulation of the excision-resynthesis processes associated with mismatch repair occurs by stimulation of the

25   ATPase activity of the hMSH2:hMSH6 heterodimer or of the ability of the heterodimer to exchange ADP for ATP. The latter stimulation can occur either by stabilizing the ADP-bound form of the heterodimer or by stimulating exchange of ADP for ATP to effect release of the heterodimer from mismatched DNA. It is thought by the inventors

that MutL homologs, such as the human MutL homologs, hMLH1, hPMS1, and hPMS2, perform these regulatory functions.

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Example 2

A Mouse Construct Nullizygous for both *msh2* and *p53* and

Methods of Making and Use Thereof

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Transgenic mice which are nullizygous for both *Msh2* and *p53* have been made, and are referred to herein as $Msh2^{-/-}p53^{-/-}$ mice. Other transgenic animals which are nullizygous for both *Msh2* and *p53*, and which particularly include mammals, especially including rodents such as mice and rats, may be made using methods analogous to those described herein and are useful in the screening methods described herein.

The development of female $Msh2^{-/-}p53^{-/-}$ mouse embryos is phenotypically arrested at approximately the 9.5 day stage, and apoptosis is induced shortly thereafter in the cells of these embryos. Male $Msh2^{-/-}p53^{-/-}$ mouse embryos are viable, but succumb to tumors significantly earlier than either $Msh2^{-/-}p53^{+/\pm}$ or $Msh2^{+/\pm}p53^{-/-}$ littermates (i.e. nullizygous *Msh2* mice or nullizygous *p53* mice, respectively). Furthermore, the frequency of microsatellite instability (MSI) in tumor tissue obtained from $Msh2^{-/-}p53^{-/-}$ mice is not significantly different than the frequency in tumor tissue obtained from $Msh2^{-/-}p53^{-/-}$ mice. Synergism in tumorigenesis and independent segregation of the MSI phenotype suggest that *Msh2* and *p53* are not genetically epistatic.

$Msh2^{-/-}p53^{-/-}$ mice are useful as models of disease or disorder states which cannot be identified in mice nullizygous for only one of *Msh2* or *p53*. Furthermore, $Msh2^{-/-}p53^{-/-}$ mice are useful for identifying compositions which affect the onset or progression of such a disease or disorder state. Thus, a $Msh2^{-/-}p53^{-/-}$ mouse is particularly useful as a model system for studying multistep tumorigenesis, apoptosis, and aging.

The materials and methods used in the experiments presented in this Example are now described.

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Generation of _Msh2^{-/-}p53^{-/-}_ Mice

Methods for making heterozygous and nullizygous _Msh2_ mice and heterozygous and nullizygous _p53_ mice have been described (de Wind et al., 1995, Cell 82:321-330; Reitmair et al., 1995, Nature Genet. 11:64-70; Donehower et al., 1992, Nature 356:215-221; Jacks et al., 1994, Curr. Biol. 4:1-7; Purdie et al., 1994, Oncogene 9:603-609).

Mice heterozygous for _Msh2_ (i.e. $Msh2^{+/-}p53^{+/+}$ mice) on a mixed C57BL/6J and 129/Ola background and mice heterozygous for _p53_ (i.e. $Msh2^{+/+}p53^{+/-}$ mice) on a mixed C57BL/6J and 129/Sv were mated to produce F1 progeny heterozygous for both genes (i.e. $Msh2^{+/-}p53^{+/-}$ mice). Heterozygous sibling F1 progeny were intercrossed to produce progeny nullizygous for both _Msh2_ and _p53_ (i.e. $Msh2^{-/-}p53^{-/-}$ mice). Mice were genotyped using _Msh2_- and _p53_- specific PCR-based assays, using methods well known in the art.

Isolation of Genomic DNA

Mouse genomic DNA was extracted from ear-notched tissue of mice and from amniotic tissue of mouse embryos at 9.5, 11.5, or 13.5 days of development, using a QIAamp Tissue Kit (Qiagen, Chatsworth, CA) according to the manufacturer's instructions.

PCR-based Genotyping of Mice

A three-primer assay specific for _Msh2_ was carried out as described (Reitmair et al., 1995, Nature Genet. 11:64-70). A four-primer assay specific for _p53_ was carried out using 50 ng of template DNA in a 50 microliter reaction mixture containing 1 unit of _Taq_ polymerase (Fisher Scientific, Malvern, PA) and 100 millimolar each of the following primers, each of which is identified with a five digit number and the sequence of each of which is listed:

_10681_ (5'-GTGTTTCATT AGTTCCCCAC CTTGAC-3'; SEQ ID NO: 7);

_10480_ (5'-ATGGGAGGCT GCCAGTCCTA ACCC-3'; SEQ ID NO: 8);

_10588_ (5'-GTGGGAGGGA CAAAAGTTCG AGGCC-3'; SEQ ID NO: 9); and

_10930_ (5'-TTTACGGAGC CCTGGCGCTC GATGT-3'; SEQ ID NO: 10).

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The amplification reaction involved 35 cycles of amplification (94°C, 15 seconds; 56°C, 30 seconds; 72°C, 1 minute) using a Perkin-Elmer GeneAmp 9600 thermal cycler. The wild-type primers, *10681* and *10480*, amplified a product of about 320 base pairs length, and the targeted allele (i.e. *p53⁻*) primers, *10588* and *10930*,

5   amplified a product of about 150 base pairs length.

The gender of embryos was determined using primers specific for the Y-chromosome gene as described (Sah et al., 1995, Nature Genet. 10:175-180). The presence of the X-chromosome was confirmed separately in all cases using the following two X-chromosome specific primers to amplify the locus *DXMIT6*:

10   5'-ACCATTCAAATTGGCAAGG-3' (SEQ ID NO: 11); and
5'-GTGGCTCGAGTTGTTTGCAG-3' (SEQ ID NO: 12).

PCR cycling conditions were as described above for *p53* genotyping, except that the annealing temperature was 53°C, rather than 56°C. The X-chromosome specific primers amplified a product of about 210 base pairs in length. All PCR amplification

15   products were resolved by electrophoresis on a 2% (w/v) agarose gel alongside a 100 base pair polynucleotide ladder standard and were visualized by ethidium bromide staining.

Timed Pregnancies

Male and female mice having a known $Msh2^{+/-}p53^{+/-}$, $Msh2^{+/-}p53^{-/-}$,

20   or $Msh2^{-/-}p53^{+/-}$ genotype were mated and each of the females was examined daily for the presence of a vaginal plug (an indicator of pregnancy which appears at about day 0.5 of embryo development). Pregnant females were sacrificed at 13.5 days, at 11.5 days, or at 9.5 days gestation. Embryos were dissected out from the pregnant females into Hank's Balanced Salt Solution (Gibco BRL, Grand Island, NY) under a dissecting

25   microscope, fixed in 4% (v/v) buffered formalin, and documented by photomicrography. Amnion was retrieved from each embryo, DNA was extracted therefrom, and the sex and genotype of each embryo was determined by PCR.

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Histology

Tissue specimens were fixed in 10% (v/v) or 4% (v/v) buffered formalin and embedded in paraffin. Histological analysis was carried out on 3 micrometer-thick sections stained with hematoxylin and eosin (H&E).

5   TUNEL Assay

Paraffin-embedded tissue sections were de-waxed and rehydrated using a graded alcohol series, using methods well known in the art. Apoptotic cells and appropriate positive and negative control samples were analyzed using the *In Situ* Cell Detection Kit, AP with NBT/BCIP, manufactured by Boehringer Mannheim

10   (Indianapolis, IN), according to the manufacturer's instructions. TUNEL-stained tissue sections were analyzed both by fluorescence microscopy and light microscopy.

Kaplan-Meier Survival

Kaplan-Meier survival probability was calculated for mice that were found dead or were sacrificed when found to be moribund. The age of the mice was

15   calculated in days. Because no mice died in the control group, confidence limits could not be determined.

Microsatellite Instability in Lymphoid Tumors

Paired ear-notch (i.e. normal) and lymphoid tumor tissues were analyzed for microsatellite instability at five chromosomal loci: *D17Mit123, D10Mit2, D6Mit59,*

20   *D4Mit27,* and *D3Mit203.* Microsatellite primer sequence pairs appropriate for amplification of these loci were obtained from the World Wide Web site of the Whitehead Institute for Genome Research (http://www.genome.wi.mit.edu), and were chosen to amplify fragments containing at least twenty dinucleotide repeat sequences. PCR amplifications were carried out in a total reaction volume of 25 µl, using 50 ng of

25   DNA as template, 100 millimolar of each primer pair and 1 unit of *Taq* polymerase (Fisher Scientific, Malvern, PA). The amplification reaction involved 35 cycles of amplification (94°C, 15 seconds; 56°C, 30 seconds; 72°C, 1 minute). Amplified products were resolved by electrophoresis on a 6.7% (w/v) denaturing polyacrylamide gel and were visualized by silver nitrate staining of the gel.

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The results of the experiments presented in this Example are now described.

Twenty-one $Msh2^{-/-}p53^{-/-}$ mice were generated from $Msh2^{+/-}p53^{+/-}$, $Msh2^{-/-}p53^{+/-}$, or $Msh2^{+/-}p53^{-/-}$ parents. When the gender of each of the twenty-one

5    $Msh2^{-/-}p53^{-/-}$ mice was examined, all were determined to be male $Msh2^{-/-}p53^{-/-}$ mice. The absence of female $Msh2^{-/-}p53^{-/-}$ offspring is highly significant ($p < 0.001$) and is unlikely to reflect the intrinsic bias for males observed in the colony from which the mice were derived, wherein the normal male:female ratio is 181:138.

The fertility of male $Msh2^{-/-}p53^{-/-}$ mice could not be determined,

10   because they succumbed to tumors before they successfully mated. However, pathological examination of the testes of the male $Msh2^{-/-}p53^{-/-}$ mice did not reveal gross abnormalities upon autopsy, and histology revealed mature spermatogenesis in all twenty-one of the male $Msh2^{-/-}p53^{-/-}$ mice. Taken together, these results suggest that $Msh2^{-/-}p53^{-/-}$ male mice are not sterile.

15   No gross morphological abnormalities were observed in $Msh2^{-/-}$ animals either *in utero* or post-natally (de Wind et al., 1995, Cell 82:321-330; Reitmair et al., 1995, Nature Genet. 11:64-70). In addition, the number of male and female $Msh2^{-/-}$ mice in the studies described herein was in accord with the expected 1:1 ratio, which suggests that male and female nullizygous $Msh2$ mice are equally viable.

20   However, a decrease in the number of live born nullizygous $p53$ mice from the expected Mendelian ratio was observed, which is qualitatively similar to previous reports, although our limited numbers did not indicate a sex bias (Sah et al., 1995, Nature Genet. 10:175-180; Nicols et al., 1995, Nature Genet. 10:181-187).

No female $Msh2^{-/-}p53^{-/-}$ mice were observed at weaning and none of

25   thirteen one-day-old pups which were found dead in the litters of mating pairs were $Msh2^{-/-}p53^{-/-}$. Thus, all female embryos nullizygous for both $Msh2$ and $p53$ died *in utero*. To determine the point in embryonic development at which these embryos died, numerous timed pregnancies were established. Because $Msh2^{-/-}p53^{-/-}$ males were not available and $Msh2^{-/-}p53^{-/-}$ females were not viable, pairs of mice, each of which mice

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was a known $Msh2^{+/-}p53^{+/-}$, $Msh2^{+/-}p53^{-/-}$, or $Msh2^{-/-}p53^{+/-}$ mouse, were mated to produce $Msh2^{-/-}p53^{-/-}$ embryos. Pregnant females were sacrificed at 9.5, 11.5, and 13.5 days of gestation, the embryos were pathologically assessed for developmental defects and the genotype and gender of each embryo were determined by PCR. The

5   results of these analyses are presented in Table 1. A total of twenty-one embryos and six resorption sites were recovered from three females at day 13.5 of gestation. Of the twenty-one 13.5 day embryos, two male $Msh2^{-/-}p53^{-/-}$ embryos and no female $Msh2^{-/-}$ $p53^{-/-}$ embryos were recovered, although a total of five $Msh2^{-/-}p53^{-/-}$ embryos were statistically expected. Two 13.5 day embryos (one male $Msh2^{+/-}p53^{-/-}$; one female

10         $Msh2^{-/-}p53^{+/-}$) displayed exencephaly, while all other 13.5 day embryos appeared normal (Sah et al., 1995, Nature Genet. 10:175-180).

Table 1

Sex and Morphological Phenotype of Timed Post-Implantation Embryos

Days Development	Resorption Sites	# of Embryos	Embryos Typed	Female $Msh2^{-/-}p53^{-/-}$		Male $Msh2^{-/-}p53^{-/-}$	
				Nor	Abnr	Nor	Abnr
e9.5	3	30	28	3	1	2	1
e11.5	11	21	17	0	4	2	0
e13.5	6	21	21	0	0	2	0
*28	-	*96	*96	*0	*0	*21	*0

20   *Refers to live-born animals at twenty-eight days following birth.

In Table 1, embryos that arrested in development, that were in resorption, or that displayed gross abnormalities were classified as abnormal (Abnr), while those embryos which were not arrested in development, were not in resorption, and did not display gross abnormalities were classified as normal (Nor). Thirteen

25   newborn pups that were found dead, none of which were $Msh2^{-/-}p53^{-/-}$, are not represented in this Table.

Twenty-one embryos and eleven resorption sites were recovered from three pregnant females at day 11.5 of gestation. Of these, complete PCR typing results were determined for seventeen embryos and one resorption site. Five embryos were

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determined to be $Msh2^{-/-}p53^{-/-}$, although eight $Msh2^{-/-}p53^{-/-}$ embryos were

statistically expected. Two of the five embryos were males that appeared

morphologically normal (one is depicted in Fig. 9A), and three of the five embryos

were females, all three of which had undergone developmental arrest, and all three of

5   which are depicted in Figures 9B, 9C, and 9D. The three female $Msh2^{-/-}p53^{-/-}$

embryos appeared opaque and somites were not visible. Based on the gross

morphology of the three female $Msh2^{-/-}p53^{-/-}$ embryos, it was estimated that they died

at 9.5 days of development. The tissue from the resorption site was typed as female

$Msh2^{-/-}p53^{-/-}$.

10   Thirty embryos and three resorption sites were recovered from pregnant

females at day 9.5 of gestation. Twenty-eight embryos and one resorption site were

successfully typed. Two embryos and a resorption site were found to be male $Msh2^{-/-}$

$p53^{-/-}$, and four embryos were typed as female $Msh2^{-/-}p53^{-/-}$. Six $Msh2^{-/-}p53^{-/-}$

embryos were statistically expected. Neither of the male $Msh2^{-/-}p53^{-/-}$ embryos

15   exhibited any gross morphological abnormality. It is likely that the male $Msh2^{-/-}p53^{-/-}$

resorption site represents a spontaneous abortion event. In one of the four female

$Msh2^{-/-}p53^{-/-}$ embryos, the anterior neural tube was not closed and the heart was not

seen to beat, which should occur around day 9 of development. These observations are

consistent with a developmental delay that could result from late fertilization or

20   implantation or alternatively, from a developmental abnormality that is apparent at day

9.5.

            Paraffin embedded tissue sections from wildtype and $Msh2^{-/-}p53^{-/-}$

female embryos, as depicted in Fig. 10, from $Msh2^{-/-}$ embryos, and from $p53^{-/-}$

embryos were examined at day 11.5 and at day 13.5. While the wildtype, $Msh2^{-/-}$, and

25     $p53^{-/-}$ embryos had clearly distinguished developmental features at day 11.5, the

arrested $Msh2^{-/-}p53^{-/-}$ female embryos contained noncohesive cells without

preservation of embryonal tissue structures. In addition, H&E stained $Msh2^{-/-}p53^{-/-}$

female embryonic tissue sections appeared to contain an large number of "blebbed"

structures typical of apoptotic cells. Furthermore, loss of nuclear hematoxylin stain

typical for necrosis was not observed in H&E stained $Msh2^{-/-}p53^{-/-}$ female embryonic tissue sections (Fig. 10, Panel B).

TUNEL staining was performed on the paraffin embedded tissue sections (Fig. 10, Panels C-F). Although wildtype (Fig. 10, Panels C and E), $Msh2^{-/-}$,

5   and $p53^{-/-}$ embryos displayed circumscribed foci of apoptotic cells characteristic of normal embryonal development, $Msh2^{-/-}p53^{-/-}$ female embryos displayed global catastrophic apoptosis (Fig. 10, Panels D and F). Furthermore, fluorescence TUNEL staining of $Msh2^{-/-}p53^{-/-}$ female embryos revealed a speckled intracellular patterning characteristic of fragmented chromatin (Fig. 10, Panel F). It was estimated that

10   between about 60% and about 90% of cells in $Msh2^{-/-}p53^{-/-}$ female embryos were undergoing visible apoptosis, as assessed by H&E and TUNEL staining.

Kaplan-Meier survival analysis was performed on a cohort of ninety-six mice, the data for which analysis are graphically presented in Fig. 11. $Msh2^{-/-}p53^{-/-}$ mice began to die of generalized lymphomas at day 53 after birth and all twenty-one

15     $Msh2^{-/-}p53^{-/-}$ mice were dead within four months of birth. In contrast, only 18% (eight of forty-four) of $Msh2^{-/-}$ littermates and 71% (five of seven) of $p53^{-/-}$ littermates were dead at the time the mice were analyzed. Thus, $Msh2^{-/-}p53^{-/-}$ mice had a significantly ($p < 0.001$) reduced median survival time of 73 days compared with the median survival time of either $Msh2^{-/-}$ mice (i.e. 200 days) or $p53^{-/-}$ mice (i.e. 149

20   days). Furthermore, all twenty-four wild-type (i.e. $Msh2^{+/\pm}p53^{+/\pm}$) littermates were alive after approximately ten months. These results indicate that $Msh2$ and $p53$ null mutations cooperatively promote tumorigenesis. $p53$ has also been shown to cooperate with a variety of other genes in mouse tumorigenesis models (Blyth et al., 1995, Oncogene 10:1717-1723; Williams et al., 1994, Cold Spring Harbor Symp. Quant.

25   Biol. 59:449-457; Williams et al., 1994, Cell 79:329-339; Donehower et al., 1995, Genes Dev. 9:882-895; Nacht et al., 1996, Genes Dev. 10:2055-2066). However, as is apparent from Fig. 11, the effect on tumor-related death of having dual null mutations of $Msh2$ and $p53$ is greater than the sum of the effects of having a single null mutation in $Msh2$ or $p53$ alone. Thus, the $Msh2^{-/-}p53^{-/-}$ mouse described herein has a

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phenotype which is significantly different from a mere combination of the phenotype of a $Msh2^{-/-}$ mouse and the phenotype of a $p53^{-/-}$ mouse.

Pathological examination of tumors showed that all twenty-one $Msh2^{-/-}$ $p53^{-/-}$ mice developed highly aggressive generalized lymphomas involving major organs. In addition, a pleomorphic sarcoma in the flank, a malignant fibrous histiocytoma of the neck, and a tubular adenoma of the small intestine were observed, while other epithelial neoplasms were not detected. The tumor spectrum of $Msh2^{-/-}$ and $p53^{-/-}$ mice appeared similar to previous observations (de Wind et al., 1995, Cell 82:321-330; Reitmair et al., 1995, Nature Genet. 11:64-70; Donehower et al., 1992, Nature 356:215-221; Jacks et al., 1994, Curr. Biol. 4:1-7; Purdie et al., 1994, Oncogene 9:603-609). The tumor spectrum of $Msh2^{-/-}p53^{-/-}$ mice differs significantly from the tumor spectrum of either $Msh2^{-/-}$ mice or $p53^{-/-}$ mice. Thus, $Msh2^{-/-}p53^{-/-}$ mice have utility different from that of either $Msh2^{-/-}$ mice or $p53^{-/-}$ mice.

Normal and tumor tissues obtained from individual $Msh2^{-/-}p53^{-/-}$ mice were examined for microsatellite instability at five loci: $D17Mit123$, $D10Mit2$, $D6Mit59$, $D4Mit27$, and $D3Mit203$. The results of these MSI studies are presented in Table 2. The frequency of MSI in tumor tissues obtained from $Msh2^{-/-}$ mice was not significantly different (p>0.05) from the frequency of MSI in tumor tissues obtained from $Msh2^{-/-}p53^{-/-}$ mice. Microsatellite instability was not observed in lymphomatous tumor tissue obtained from the seven $p53^{-/-}$ mice examined. The observation that $Msh2^{-/-}p53^{-/-}$ mice developed earlier onset of tumor-related disease, combined with the observed separate segregation of the MSI phenotype with the $Msh2$ allele, suggests that $Msh2$ and $p53$ are not genetically epistatic.

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Table 2

The Frequency of Microsatellite Instability in $p53^{-/-}$, $Msh2^{-/-}$, and

$Msh2^{-/-}$ $p53^{-/-}$ Mice

Genotype Tumor / Normal Pairs	Tumors Examined (n)	MSI at ≥1 Locus	MSI at ≥2 Loci	MSI at ≥3 Loci
$p53^{-/-}$	7	0 (0%)	0 (0%)	0 (0%)
$Msh2^{-/-}$	8	6 (75%)	4 (50%)	3 (38%)
*$Msh2^{-/-}p53^{-/-}$	21	17 (81%)	14 (67%)	12 (57%)

*Because female $Msh2^{-/-}p53^{-/-}$ mice died during embryonic development, this refers to only male $Msh2^{-/-}p53^{-/-}$ mice.

It is remarkable that female $Msh2^{-/-}p53^{-/-}$ mouse embryos underwent global developmental arrest and that widespread apoptosis of the cells of such embryos occurred around day 9.5 of development. That these embryos underwent implantation and gastrulation strongly suggests that they are capable of executing the earlier stages of embryogenesis. The arrested phenotype is reminiscent of that described for a small proportion of female $p53^{-/-}$ mice (Sah et al., 1995, Nature Genet. 10:175-180). However, unlike $p53^{-/-}$ mice, no normal female $Msh2^{-/-}p53^{-/-}$ mice or embryos were observed beyond 9.5 days of embryonic development. This observation supports the conclusion that the female embryonic lethality of $Msh2^{-/-}p53^{-/-}$ mice is highly penetrant. In addition, none of the female $Msh2^{-/-}p53^{-/-}$ embryos displayed the exencephaly that characterized the $p53^{-/-}$ mice (Sah et al., 1995, Nature Genet. 10:175-180). Furthermore, while there was no difference in apoptosis observed in developing $p53^{-/-}$ mouse embryos, global catastrophic apoptosis was clearly observed in all the $Msh2^{-/-}p53^{-/-}$ female mouse embryos examined at day 9.5 of development. These results suggest that female $Msh2^{-/-}p53^{-/-}$ mice succumb at an earlier stage and by an entirely different pathology than $p53^{-/-}$ mice.

Without being bound to any particular theory, the lethality observed in female $Msh2^{-/-}p53^{-/-}$ mouse embryos is consistent with the following explanation. In the female embryonic lineage, dosage compensation is achieved by random X

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chromosome inactivation around the time of gastrulation, at which time intense embryonic cellular proliferation and apoptosis promote embryonic differentiation (Lyon, 1961, Nature 190:372-373; Rastan, 1994, Curr. Opin. Genet. Dev. 4:292-297; Theiler, 1972, In: The House Mouse Development and Normal Stages from

5 Fertilization to 4 Weeks of Age, Springer-Verlag, New York, p. 168). The global apoptotic effect need not occur coincidentally with X chromosome inactivation. The full effect of dysregulation may only become apparent after a number of cell divisions when the embryo undergoes a further burst of proliferation during embryonic 'turning' between 8 and 9.5 days.

10   It has been shown that the inactivated X chromosome replicates late in S phase (Taylor, 1960, J. Biophys. Biochem. Cytol. 7:455-464; Tagaki, 1974, Exp. Cell. Res. 86:127-135). In addition, cells deficient in $p53$ have been shown to be defective for damage-induced G_1/S checkpoint arrest, and cells that are deficient in MMR have been shown to be deficient for damage-induced G_2/M checkpoint arrest (Baker et al.,

15 1990, Science 249:912-915; Diller et al., 1990, Mol. Cell. Biol. 10:5772-5781; Lin et al., 1992, Proc. Natl. Acad. Sci. USA 89:9210-9214; Hawn et al., 1995, Cancer Res. 55:3721-3725; Marra et al., 1996, Oncogene 13:2189-2196). Thus, female-specific $Msh2^{-/-}p53^{-/-}$ embryo lethality may result from dysregulation of damage-induced arrest checkpoint control, wherein such dysregulation is caused by a deficiency of both

20 $p53$ and $Msh2$, and whereby such dysregulation results in an inability of $Msh2^{-/-}p53^{-/-}$ cells to arrest cell division and repair damage introduced into the late replicating inactive X chromosome. Such damage could take the form of non-replicated regions or chromosomal fragments that have resulted from inappropriate cell division prior to the completion of inactive X chromosome replication. Fragmented, reactivated, or

25 otherwise altered inactive X chromosomes may then lead to global catastrophic cellular failure, developmental arrest, and apoptosis. Furthermore, the observation that the highest levels of p53 mRNA are detected in wild-type embryos between 9 and 11 days of development suggests an important role for p53 protein within this time frame (Rogel et al., 1985, Mol. Cell. Biol. 5:2851-2855).

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<u>Example 3</u>

<u>A Discussion of hMSH2:hMSH6 Heterodimers in the Context of</u>

<u>Mismatch Repair, Molecular Switches, and Signal Transduction</u>

The foundation of molecular switches in biology is grounded in

5   translation elongation and cellular signal transduction. In these systems, guanine

nucleotide-bound proteins (G-proteins) produce the ON and OFF signaling states that

act as gates for downstream biochemical processes. Experimental results described

herein, in view of the results of studies by others, suggest that a similar molecular

switch relies on adenine nucleotide-bound forms (A-proteins) to produce an ON and

10   OFF signaling state related to mismatched DNA repair and possibly to other processes.

In the field of signal transduction, the concept of a molecular switch is elementary,

while the biochemical processes of DNA repair appear foreign. Similarly, the field of

DNA repair recognizes the complex machinery required for DNA manipulation events,

but regards biochemical signaling processes as essential cellular input which is outside

15   the genome juggernaut.

<u>Genetics of Mismatch Repair</u>

There are at least three ways in which mismatched nucleotides arise in

DNA. Physical or chemical damage to the DNA and its precursors, such as de-

amination of 5-methyl-cytosine, can give rise to mismatched bases (Friedberg, 1990,

20   <u>DNA Repair</u> W.H. Freeman Co., New York). Misincorporation of nucleotides during

DNA replication can yield mismatched base pairs as well as the insertion and deletion

of nucleotides (for review see: Kolodner, 1996, Genes Dev. 10:1433-1442; Modrich,

1989, J. Biol. Chem. 264:6597-6600; Modrich, 1997, J. Biol. Chem. 272:24727-

24730). Genetic recombination produces regions of heteroduplex DNA which may

25   contain mismatched nucleotides when such heteroduplexes result from the pairing of

two different parental DNA sequences (Holliday, 1964, Genet. Res. 5:282-304).

Mismatched nucleotides produced by each of these mechanisms are known to be

repaired by enzyme systems that are both specific and overlapping (Friedberg, 1990,

<u>DNA Repair</u>, W.H. Freeman Co., New York).

The most extensively studied system for mismatch repair (MMR) is the
DNA adenine methylation (Dam)-instructed pathway of *Escherichia coli* (Modrich,
1989, J. Biol. Chem. 264:6597-6600; Modrich and Lahue, 1996, Annu. Rev. Biochem.
65:101-133). The Dam-Instructed pathway promotes a long-patch (approximately 2

5   kilobase pair) excision repair reaction which is genetically dependent on the *mutH,
mutL, mutS, and mutU (uvrD)* gene products. Discrimination of the newly replicated
DNA strand from the original template DNA strand is dependent on transient under-
methylation of the adenine nucleotide within GATC Dam sequences. The MutHLS
pathway appears to be the most active MMR pathway in *E. coli* and is known to both

10   increase the fidelity of DNA replication as well as to act on recombination
intermediates containing mis-paired bases (Fishel et al., 1983, UCLA Symp. Mol. Cell.
Biol. New Series 11:309-324; Fishel et al., 1986, J. Mol. Biol. 188:147-157).

      Homologs of prokaryotic MutS and MutL proteins have been identified
in nearly every organism with the exception of Archaea (Fishel et al., 1997, Curr. Opin.

15   Genet. Dev. 7:105-113; Kolodner, 1996, Genes Dev. 10:1433-1442). At present, there
are 41 MutS homologs and 21 MutL homologs in the NCBI database. In *S. cerevisiae*,
six *MutS* homologs (MSH1 - MSH6) and three *MutL* homologs (MLH1, MLH2,
PMS1) have been identified. In human cells, a nearly identical set of five MutS
homologs (hMSH2 - hMSH6) and three MutL homologs (hMLH1, hPMS1, and

20   hPMS2) are known (Acharya et al., 1996, Proc. Natl. Acad. Sci. USA 93:13629-13634;
Bronner et al., 1994, Nature 368:258-261; Burns et al., 1994, Genes Dev. 8:1087-1105;
Fishel et al., 1993, Cell 75:1027-1038; Fujii et al., 1989, J. Biol. Chem.
264:10057-10064; Hollingsworth et al., 1995, Genes Dev. 9:1728-1739; Kramer et al.,
1989, J. Bacteriol. 171:5339-5346; Linton et al., 1989, Mol. Cell. Biol. 9:3058-3072;

25   Mankovich et al., 1989, J. Bacteriol. 171:5325-5331; New et al., 1993, Mol. Gen.
Genet. 239:97-108; Nicolaides et al., 1994, Nature 371:75-80; Palombo et al., 1995,
Science 268:19121-19914; Prolla et al., 1994, Mol. Cell. Biol. 14:407-415; Reenan et
al., 1992, Genetics 132:963-973). Yet, with the exception of gram-negative bacteria,
there do not appear to be homologs of MutH. Thus, the mechanism of strand

discrimination in even close relatives of *E. coli*, the gram-positive bacteria, remains a mystery. The multiple MutS and MutL homologs have been found to participate in the diverse activities of nuclear (MSH2, MSH3, MSH6, MLH1, PMS1) and organellar (MSH1) post-replication mismatch repair as well as having distinct meiotic functions

5   (MSH4, MSH5) (Fishel et al., 1997, Curr. Opin. Genet. Dev. 7:105-113; Kolodner, 1996, Genes Dev. 10:1433-1442).

Biochemistry of Mismatch Repair

           Purification and reconstitution studies by Modrich and colleagues have led to a biochemical model for post-replication mismatch repair in *E. coli*. The

10   reconstituted system requires the MutH, MutL, MutS and UvrD (helicase II) proteins along with DNA polymerase III holoenzyme, DNA ligase, single-stranded DNA binding protein (SSB) and one of the single-stranded DNA exonucleases, ExoI, ExoVII or RecJ (Cooper et al., 1993, J. Biol. Chem. 268:11823-11829; Grilley et al., 1989, J. Biol. Chem. 264:1000-1004; Lahue et al., 1989, Science 245:160-164; Lu et al., 1983,

15   Proc. Natl. Acad. Sci. USA 80:4639-4643; Su et al., 1986, Proc. Natl. Acad. Sci. USA 83:5057-5061; Welsh et al., 1987, J. Biol. Chem. 262:15624-15629). In this widely held biochemical model, initiation of a MMR event occurs when MutS recognizes and binds mis-paired nucleotides that result from polymerase misincorporation errors (Su et al., 1986, Proc. Natl. Acad. Sci. USA 83:5057-5061). It is suggested that MutS

20   mismatch binding is followed by interaction with the MutL protein (Grilley et al., 1989, J. Biol. Chem. 264:1000-1004), which has been proposed to accelerate an ATP-dependent translocation of the MutS-MutL complex (Allen et al., 1997, EMBO J. 16: 4467-4476) to a hemi-methylated GATC Dam site bound by MutH (Welsh et al., 1987, J. Biol. Chem. 262:15624-15629). The MutS-MutL complex then stimulates an

25   intrinsic endonuclease activity of MutH which results in a specific strand scission on the non-methylated newly replicated DNA strand (Cooper et al., 1993, J. Biol. Chem. 268:11823-11829; Lahue et al., 1989, Science 245:160-164; Welsh et al., 1987, J. Biol. Chem. 262:15624-15629). This strand scission directs one of three single-stranded exonucleases (RecJ, Exo I, ExoVII) to degrade the newly replicated strand, which is

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then re-synthesized by the PolIII holoenzyme complex (Lahue et al., 1989, Science 245:160-164). The net result is a strand-specific mismatch repair event which can be bidirectional. Many of the genetic studies performed with this system appear to support this biochemical interpretation. For example, *mutH, mutL,* and *mutS* bacteria

5   exhibit a mutator phenotype that is presumed to be the result of the increased probability of misincorporation errors leading to mutations (Demerec et al., 1957, Carnegie Inst. Wash. Yearbook 370:390-406; Hill, 1970, Mutat. Res. 9:341-344; Miyake, 1960, Genetics 45:755-762; Siegel et al., 1967, J. Bacteriol. 94:38-47). However, not all predictions arising from this model agree with the genetic results. For

10   example, *recJ exoI exoVII* bacteria do not appear to exhibit a mutator phenotype (Harris et al., 1998, J. Bacteriol. 180:989-993), suggesting that there may be other exonuclease(s) or mechanism(s) involved in the mismatch repair process.

Functions for the Mismatch Repair Proteins

An activity exhibited by mismatch repair proteins is the specific mis-

15   pair binding activity ascribed to MutS homologues (Acharya et al., 1996, Proc. Natl. Acad. Sci. USA 93:13629-13634; Chi et al., 1994, J. Biol. Chem., 269:29984-29992; Drummond et al., 1995, Science 268:1909-1912; Fishel et al., 1994, Science 266:1403-1405; Gradia et al., 1997, Cell 91:995-1005; Marsischky et al., 1996, Genes Dev. 10:407-420; Su et al., 1986, Proc. Natl. Acad. Sci. USA 83:5057-5061). A clear

20   function of the MutL homologs has, until the present invention, not been clear. Classification of MutS and MutL homologs is based on the recognition of highly conserved regions of amino acid identity. The most highly conserved region of the MutS homologs is confined to a region of approximately 150 amino acids that encompass a helix-turn-helix domain associated with a Walker-A adenine-nucleotide

25   and magnesium binding motif. Such motifs were described by Walker et al. (1982, EMBO J. 1:945-951). This adenine nucleotide binding domain constitutes 100% of the identity between the known MutS homologs (Fishel et al., 1997, Curr. Opin. Genet. Dev. 7:105-113). Purified bacterial, yeast, and human MutS homologs exhibit an intrinsic low-level ATP hydrolytic (ATPase) activity (Alani et al., 1997, Mol. Cell.

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Biol. 17: 2436-2447; Chi et al., 1994, J. Biol. Chem., 269:29984-29992; Gradia et al., 1997, Cell 91:995-1005; Haber et al., 1991, EMBO J 10:2707-2715). This ATPase activity is likely to be important for the function of the MutS homologs, as evidenced by the observation that mutation of a conserved lysine residue in the adenine nucleotide

5   binding domain results in a dominant mutator phenotype in both bacteria and yeast (Alani et al., 1997, Mol. Cell. Biol. 17: 2436-2447; Haber et al., 1991, EMBO J. 10:2707-2715).

The most widely held model for MMR suggests MutS mis-pair binding is followed by MutL association that results in an energy dependent translocation of

10   this complex to a hemi-methylated Dam site occupied by the MutH protein. In retrospect, this appears to have been a simplistic view since the rate of ATP hydrolysis ($k_{cat} \simeq 10 \text{ min}^{-1}$) is unlikely to be sufficient to drive mechanical translocation the, on average, several hundred to thousand nucleotides required to encounter a MutH bound hemimethylated site. For example, if one ATP was required to translocate one

15   nucleotide, as the most well accepted mechanism suggests, then it would take 25-100 minutes to encounter a MutH on average. Yet, re-methylation of the transiently hemimethylated Dam sites has been found to occur within 0.1 to 3 minutes of passage of the replication fork (Campbell et al., 1990, Cell 62:967-979). While the ATPase activity could in theory be significantly faster *in vivo*, no stimulatory factor has been

20   identified to date in spite of an extensive search. In addition, the prevailing mechanism does not adequately account for MutL function nor the highly conserved domains recognized between MutL homologs from bacteria to man (regions containing 100% identity in 21 homologs).

The hMSH2-hMSH6 Molecular Switch

25   As described herein in Example 2 and elsewhere, human MutS homolog dimers, such as the hMSH2:hMSH6 heterodimer, function as molecular switches responsible for the timing of mismatch repair, as illustrated in Figure 7. This conclusion is based on the observations that:

1) The ADP-bound heterodimer has high affinity for mismatched nucleotides;

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2) exchange of ADP for ATP results in release of the heterodimer from mismatched duplex DNA in the absence of hydrolysis;

3) release of the heterodimer from mismatched duplex DNA occurs by hydrolysis-independent diffusion off the ends of the short oligonucleotides used in the experiments described in Example 2, as confirmed by the experiments described in Example 4 herein; and

4) hydrolysis of ATP results in recovery of the mismatch-binding competent ADP-bound heterodimer.

The rate-limiting step and the ultimate control of the hMSH2:hMSH6 molecular switch is likely to be ADP→ATP exchange, which is exceedingly inefficient in the absence of mismatched duplex DNA. The characteristics of the hMSH2:hMSH6 heterodimer appear analogous to the characteristics of G-protein mediators of seven-transmembrane (7-TM) domain receptor signaling such as that used by the β-Adrenergic and Rhodopsin Receptors and the prototypical oncoprotein/G-protein Ras (Tocque et al., 1997 Cell Signal. 9:153-158). More specifically, the observation that the hMSH2:hMSH6 heterodimer is induced to exchange ADP for ATP in the presence of mismatched duplex DNA and then dissociates from the mismatched portion of the duplex DNA to transduce a signal, is analogous to the observation that ligand binding by 7-TM receptors induces associated G-proteins to exchange GDP→GTP and dissociate from the receptor to transduce a signal.

These similarities suggest two related models for mismatch repair that are fundamentally different from all previously suggested models. These models are each based on the concept that MutS and its homologs are a novel type of molecular switch which determines the timing and/or appropriate assembly of repair components. The apparent affinity of the hMSH2:hMSH6 heterodimer for mismatched duplex DNA ($Kd \cong 2\text{-}20$ nanomolar) suggests that a single mismatch in a human cell should be efficiently recognized and bound. Furthermore, binding of the hMSH2:hMSH6 heterodimer to mismatched duplex DNA is slightly stabilized in the presence of ADP. We would propose two non-exclusive models.

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In the first model, tight binding of the ADP-bound form of the hMSH2:hMSH6 heterodimer to mismatched duplex DNA acts as a flag for the assembly or nearby localization of DNA excision repair components. When the complete excision repair complex is assembled, exchange of ADP for ATP is triggered

5   and the hMSH2:hMSH6 heterodimer is released from the mismatched portion of the duplex DNA, thus signaling exonucleolytic excision and resynthesis of the region containing the mismatched nucleotide. Once released from the mismatched portion of the duplex DNA, the intrinsic ATPase activity of hMSH2-hMSH6 hydrolyzes bound ATP, resulting in a form that is once again competent for mis-pair binding.

10   In the second model, recognition of mismatched duplex DNA by the ADP-bound form of the hMSH2:hMSH6 heterodimer provokes ADP→ATP nucleotide exchange. ATP-hydrolysis-independent DNA-associated diffusion of the hMSH2:hMSH6 heterodimer away from the mismatch portion of the duplex DNA to the assembled (or partially assembled) DNA mismatch repair complex. Activation of

15   these components by the confederation of the ATP-bound form of the hMSH2:hMSH6 heterodimer either engages the repair process (signaling the timing of mismatch repair as above) or triggers assembly of the remaining DNA mismatch repair components. This activation event results in release of the hMSH2:hMSH6 heterodimer from the duplex DNA, hydrolysis of ATP bound to the hMSH2:hMSH6 heterodimer, and

20   recycling of the form of the hMSH2:hMSH6 heterodimer capable of associating with mismatched duplex DNA. An advantage of this second model is that the hMSH2:hMSH6 heterodimer remains associated with the DNA in an activated-form, poised to transduce the mismatch signal to any nearby mismatch repair components.

As a free protein complex, the hMSH2:hMSH6 heterodimer does not

25   efficiently exchange ADP remaining after hydrolysis of ATP bound thereto, providing a long-term mismatch recognition-competent molecule. A key difference in the mismatch repair models described above and those previously proposed, is the concept that ATP hydrolysis is not required to physically transduce the mismatch binding signal to downstream DNA mismatch repair components. Instead, ATP hydrolysis is required

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only to recycle the mis-pair recognition component (i.e. the hMSH2:hMSH6 heterodimer). Without wishing to be bound by any particular theory, it is thought that the signal state of the hMSH2:hMSH6 heterodimer is related to the conformational state of the heterodimer, which in turn is related to whether ADP or ATP is bound

5   thereto.

One of the most important observations concerning G-proteins is their regulation by associated proteins (Bokoch et al., 1993, FASEB J. 7: 750-759). There are two halves to the GTPase cycle: γ-phosphate hydrolysis and GDP$\rightarrow$GTP nucleotide exchange. Both of these steps can be regulated either by inhibition or acceleration of

10   these partial reactions. For example, the Ras protein has an remarkably sluggish intrinsic GTPase activity (Trahey et al., 1987, Mol. Cell. Biol. 7:541-544), which can be accelerated at least 104-to 105-fold by a GTPase Activating Protein (GAP) (Trahey et al., 1987, Science 238:542-545). In addition, there are other Regulators of G-Protein Signaling (RGS) that singularly accelerate GTP γ-phosphate hydrolysis, and

15   GDP$\rightarrow$GTP exchange stimulators (GES) and guanine dissociation inhibitors (GDI) that singularly affect nucleotide exchange (Dohlman et al., 1997, J. Biol. Chem. 272:3871-3874; Quilliam et al., 1995, Bioessays 17:395-404; Tocque et al., 1997, Cell Signal 9:153-158). It has been discovered herein that MutL homologs perform analogous functions (i.e. accelerate ATP γ-phosphate hydrolysis, and ADP$\rightarrow$ATP

20   exchange) with respect to MutS homologs.

Biological Switches and the Second Law of Thermodynamics

One could argue that the concept of a singular ON or OFF state in a molecular switch might violate the second law of thermodynamics. This law requires that biochemical systems transit one state to the other by a series of microscopically

25   reversible steps. This idea is based in statistical mechanics as it is applied to a system at equilibrium - which must be applied a priori to enzyme catalyzed biological processes. It is easy to visualize the origins of the principle of microscopic reversibility by considering the consequences were it NOT true. For example, if the rate of A$\rightarrow$B were greater than B$\rightarrow$A at equilibrium, each of the rates B$\rightarrow$C, C$\rightarrow$D, and D$\rightarrow$A would

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also have to be greater than their reverse rates in order to prevent build-up of the
concentration of any species, which is not permitted at equilibrium. In this case there
would be a preferred direction-of-operation of the reaction cycle. Such a spontaneous
cycle in a system at equilibrium (i.e. an engine that spontaneously produces work) is

5   not consistent with the drive toward maximum entropy contained in the second law of
thermodynamics.

There is no violation of the second law of thermodynamics if the transit from an
OFF to ON state (or visa versa) occurs reversibly. The molecular basis for this type of
microscopic reversibility can be visualized for the MutS dimer and G-protein switches

10   as reversible nucleotide-binding as well as intermediate protein conformational changes
that occur while transiting the extreme states. It is these conformational transitions that
determine interaction with effectors which is ultimately accounted for by the hydrolysis
of NTP. More significantly, one can experimentally affect the equilibrium of each state
by altering the ratio of NDP/NTP in the absence of any hydrolysis, as indicated in

15   Figure 4B. It is also important to note that microscopic reversibility has been directly
demonstrated for the "gated" maxi K^+ ion pump, which is a molecular switch
controlled by similar conformational transitions (Song et al., 1994, Biophys. J.
67:91-104). Thus, molecular switches are both reversible and, at equilibrium, clearly
preserving a fundamental tenant of thermodynamics.

20   Similarities Between Signal Transduction and DNA Metabolism

The use of controlled molecular switches appears to pervade all aspects
of biology. From the standpoint of DNA metabolism, switch controlled processes
appear mechanistically sensible. It is well known that the cellular components which
perform replication, recombination, repair, and chromosome segregation are very large

25   and composed of multiple subunits (Alberts, 1998, Cell 92:291-294). Analogous to an
assembly-line for an automobile or an airplane, the assembly of DNA metabolic
machines must be done precisely and in a specific order to ensure appropriate function.
A series of well defined switches could logically control the progression of such an
ordered assembly process.

The same type of switch-controlled cascade events that transduce cellular signals may also control DNA metabolic events. An important difference between these switches is the identity of the nucleotide that induces the conformational transitions associated with signaling. At the moment the general rule seems to be that
5   guanine nucleotides are involved in cellular signaling events and adenine nucleotides are involved in DNA metabolic signaling events.

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Example 4

Interactions of hMSH2 with hMSH3 and of hMSH2 with hMSH6:

Examination of Mutations Associated with HNPCC

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10   In the experiments described in this Example, mutations in the human mismatch repair protein hMSH2 were determined to co-segregate with the occurrence in individuals afflicted with hereditary non-polyposis colorectal cancer (HNPCC). As described herein, hMSH2 forms specific mis-pair binding complexes with hMSH3 and hMSH6. These protein interactions were further characterized by mapping the contact
15   regions between the monomers of the hMSH2:hMSH3 and hMSH2:hMSH6 heterodimers.

The results described in this Example demonstrate that there are at least two distinct regions of monomer:monomer interaction in both hMSH2:hMSH3 and hMSH2:hMSH6 heterodimers. The same regions of the hMSH2 monomer interact
20   with regions of both the hMSH3 monomer and the hMSH6 monomer. Furthermore, there is a coordinated linear orientation of these regions, by which is meant that the amino-terminal region of hMSH2 associates with the amino-terminal of either hMSH3 or hMSH6 and the carboxy-terminal region of hMSH2 associates with the carboxy-terminal region of either hMSH3 or hMSH6. Several missense alterations of hMSH2
25   obtained from HNPCC kindreds were examined and were determined to occur within the consensus monomer:monomer interaction regions. None of these missense mutations prevented monomer:monomer interaction. These data support the idea that an altered interaction of hMSH2 with hMSH3 or an altered interaction of hMSH2 with hMSH6 is unlikely to be causative of HNPCC.

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In the experiments described in this Example the regions of monomer:monomer interaction were ascertained for hMSH2:hMSH3 and hMSH2:hMSH6 heterodimers. Two distinct interaction regions were identified for hMSH2:hMSH3 heterodimers and for hMSH2:hMSH6 heterodimers. The interaction

5   regions of hMSH2 with either hMSH3 or hMSH6 appeared to be identical. Several missense mutations of hMSH2 were constructed. These mutations have been reported by others to co-segregate with HNPCC. None of these alterations affected the interactions between hMSH2 and either hMSH3 or hMSH6 heterodimers.

The materials and methods used in the experiments presented in this

10   Example are not described.

<u>Reagents and Enzymes</u>

Restriction endonucleases were obtained from New England Biolabs (Beverly, MA). PCR reactions were performed using the High Fidelity PCR Kit obtained from Boehringer Mannheim (Mannheim, Germany). Oligonucleotides were

15   synthesized using an Applied Biosystems (Foster City, CA) model 3948 nucleic acid synthesis and purification system. DNA plasmid constructs were purified using Qiagen (Hilden, Germany) DNA purification kits. *In vitro* transcription and translation (IVTT) reactions were performed using the Promega (Madison, WI) TNT™ Coupled Rabbit Reticulocyte Lysate System. Radiolabeled ^{35}S methionine was used to label proteins

20   and was obtained from Dupont NEN (Wilmington, DE). Glutathione linked (GST) agarose beads were purchased from Sigma (St. Louis, MO).

<u>Subcloning of hMSH2 and hMSH3</u>

The cloning of hMSH2, hMSH3, and hMSH6 cDNAs and subcloning into pET expression vectors (obtained from Novagen) has been previously described

25   (Acharya et al., 1996, Proc. Natl. Acad. Sci. USA 93:13629-13634). In this study, we used a HeLa cDNA clone of hMSH3 (Gen Bank Accession U61981).

GST fusion proteins were synthesized using the pGEX system (Pharmacia, Sweden). For ease of cloning, plasmid pGEX-4T-2 was modified as follows. The vector DNA was digested using *Eco*RI and *Bam*HI restriction

endonucleases and purified by gel electrophoresis. A double-stranded linker
oligonucleotide comprising a polynucleotide having the nucleotide sequence SEQ ID
NO: 13 and a polynucleotide having the nucleotide sequence SEQ ID NO: 14 was
ligated into the vector. SEQ ID NO: 13 is 5'-GATCCGAGAA CCTGTACTTC

5   CAGGGACATA TGGCCATGGG TACCG-3'. SEQ ID NO: 14 is 5'-AATTCGGTAC
CCATGGCCAT ATGTCCCTGG AAGTACAGGT TCTCG-3'. The vector is herein
referred to as pGEX-SG1 and permitted subcloning using *Nde*I and *Nco*I restriction
endonuclease sites in which the ATG initiation codon within each site was in frame
with the GST moiety. Vector pGEX-SG1 also contained a TEV protease site just

10   upstream of the *Nde*I and *Nco*I sites.

Construction of hMSH2 truncation mutations

        The hMSH2 deletion mutants were constructed using known PCR
truncation mutagenesis methods. 'Forward' primers were generated by adding a
polynucleotide homologous with six codons corresponding to the desired 3'-end of

15   *Msh2*, starting with a codon having a guanine residue in the 5'-position and adding the
17 nucleotides immediately 3'- with respect to that residue, to the 3'-end of a
polynucleotide having the nucleotide sequence 5'-GCGGATCCCA TGG-3' (SEQ ID
NO: 15). 'Reverse' primers were generated by adding a polynucleotide homologous
with the 18 nucleotides of the complementary strand corresponding to the six codons of

20   desired 5'-end of *Msh2* to the 3'-end of a polynucleotide having the nucleotide sequence
5'-GGAGGATCCC TA-3' (SEQ ID NO: 16). Using a forward and reverse primer, a
PCR reaction was performed using pET3d-hMSH2 as template DNA. The PCR
product and pET24d were digested with *Nco*I and *Bam*HI, purified by gel
electrophoresis, and ligated together.

25   To make truncated peptides containing an internal deletion, pET
24d-hMSH2 (which did not encode amino acid residues 700-800 of hMSH2) was
generated by performing PCR on hMSH2 using a pair of polynucleotide primers
having sequences 5'-GCGGATCCCA TGGCAGAAGT GTCCATTGTG-3' (SEQ ID
NO: 17) and 5'-GGAGGATCCC ATATGTAGAT TATTAACAGT TGG-3' (SEQ ID

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NO: 18). The amplification product and pET24d were digested using *Nco*I and *Bam*HI, and the digested products were purified by gel electrophoresis and ligated together. The resulting vector permitted ligation of fragments using *Nde*I and *Bam*HI. 'Forward' primers were designed using the first 18 nucleotides of the desired 3'-end of *msh2*

5   ligated to the 3'-end of a polynucleotide having the sequence 5'-GGCGGTATCC ATATG-3' (SEQ ID NO: 19). The reverse primer was the same as the one described earlier in this Example. PCR fragments were ligated into this vector using *Nde*I and *Bam*HI. Site directed mutagenesis of hMSH2 was performed using overlap PCR, as described (Kallal et al., 1997, Mol. Cell. Biol. 17:2897-2907). All of the site directed

10   mutations were completely sequenced using a Perkin Elmer ABI Sequencer with XL upgrade (Perkin Elmer Cetus, Norwalk, CT).

Construction of hMSH3 and hMSH6 truncation mutations

hMSH3 and hMSH6 truncation constructs were created using a method analogous to that used to generate to the hMSH2 deletion mutants. 'Forward' primers

15   were generated using the same method described for designing hMSH2 'forward' primers for hMSH2 mutations having truncations. The reverse primers were generated using the same method described for designing hMSH2 'reverse' primers for hMSH2 mutations having either truncations or internal deletions, except that the polynucleotide had the sequence 5'-GGCATACTCG AGCTA-3' (SEQ ID NO: 20), instead of SEQ ID

20   NO: 16. The PCR amplification product was subcloned into either pET24d or pGEX-SG1.

pET24d-hMSH3 (which did not encode amino acid residues 800-990 of hMSH3) was constructed by performing PCR using *msh3* and a pair of polynucleotide primers having sequences 5'-GCGGATCCCA TGGATTTTCT AGAGAAATTC-3'

25   (SEQ ID NO: 21) and 5'-GGACGCGTCG TCGACCTAAC CGGTATCTCT GATGAAATAC TC-3' (SEQ ID NO: 22). The amplified product and pET24d were digested using restriction endonucleases *Nco*I and *Sal*I and subcloned. This vector permitted ligation of inserts using restriction endonucleases *Age*I and *Xho*I. Forward primers were generated by ligating six codons corresponding to the desired 3'-end of

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msh3 to a polynucleotide having the sequence 5'-GCGGTGACCG GT-3' (SEQ ID NO: 23). Reverse primers were generated as described earlier, only homologous with the non-coding strand of *msh3*. PCR was performed, and the amplified products were ligated.

5   In order to avoid errors introduced by random PCR mutagenesis, all PCR amplification products were either completely sequenced or the experiments were conducted using two separately isolated PCR products.

GST Fusion Protein Interaction assay

          An overnight culture of *E. coli* XL-blue cells which harbored
10   pGEX-hMSH(X) (i.e. 'X' being 2, 3, or 6) was grown in LB with 50 milligrams per milliliter ampicillin. 50 milliliter of Luria broth containing ampicillin was inoculated with 1 milliliter of the overnight culture, and the culture was incubated until the optical density, as assessed at 600 nanometers, was about 0.5. IPTG was added to a final concentration of 0.1 millimolar, and the culture container placed in a shaker at 30°C for
15   2 hours to generate induced cells. Induced cells were pelleted and resuspended in 800 milliliters of phosphate buffered saline (Boehringer Mannheim, Germany) containing protease inhibitors (0.5 millimolar PMSF, 0.8 milligrams per milliliter leupeptin, 0.8 milligrams per milliliter pepstatin, and 0.1 millimolar EDTA). Lysozyme was added to a concentration of 1 milligram per milliliter, and the mixture was incubated on ice for
20   30 minutes. Triton X-100 and dithiothreitol were added to final concentrations of 0.2% (v/v) and 2 millimolar, respectively. The lysate was frozen and thawed twice to completely lyse the cells. DNase (Boehringer Mannheim, Germany) was added to a final concentration of 20 micrograms per milliliter, and the lysate was incubated on ice for an additional 20 minutes. Cell debris was removed by centrifuging the lysate at
25   14,000 rpm in a refrigerated Eppendorf (Model 5402) centrifuge for 30 min. The supernatant was transferred to a new microfuge tube which contained rehydrated GST-agarose beads in a proportion whereby approximately 10-50 nanograms of protein were present for every 25 microliters of beads that were present. GST-fusion protein levels were quantified as described herein. The lysate was incubated with the GST-agarose

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beads at 4°C on a rocking platform. After rocking for 1-2 hours, the incubation mixture was centrifuged at 1000 rpm in an Eppendorf microfuge for 30 seconds, the supernatant removed, and the beads were gently resuspended in 500 milliliters of Binding Buffer. Binding Buffer consisted of 20 millimolar Tris, pH 7.5, 10% (v/v)

5 glycerol, 150 millimolar NaCl, 5 millimolar EDTA, 1 millimolar DTT, 0.1% (v/v) Tween 20, 0.75 milligrams per milliliter BSA, 0.5 millimolar PMSF, 0.8 milligrams per milliliter leupeptin, and 0.8 milligrams per milliliter pepstatin. The centrifugation and re-suspension was repeated three times to wash the beads substantially free of non-specific lysate proteins. Suspended beads were added to a 14 milliliter sterile

10 polypropylene tube, diluted with Binding Buffer to approximately 50 microliters of packed glutathione beads per milliliter and incubated at 4°C on a rocking platform for 30 minutes in order to allow BSA to coat the beads. 500 milliliters of these coated GST-fusion protein associated glutathione beads, which comprised about 10-50 nanograms of bound GST-fusion protein, was then aliquoted into 1.5 milliliter

15 microfuge tubes. GST-fusion protein expression levels were quantitated by Coomassie Brilliant Blue staining of protein separated by SDS-PAGE gels, using BSA as a standard.

In vitro transcription and translation (IVTT) reactions involving ^{35}S-Methionine were performed with pET-hMSH(Y) (i.e. where 'Y' was 2, 3, or 6)

20 using purified DNA according to the manufacturers recommendations. IVTT reactions were pre-run to determine the relative molar concentration of each construct. This value was calculated using the specific activity of ^{35}S-Methionine, correcting for the number of methionine residues in each IVTT construct and using SDS-PAGE and a Molecular Dynamics PhosphorImager device equipped with ImageQuant software

25 (Sunnyvale, CA). Up to 10 microliters of the IVTT protein was added to each tube such that each sample contained an approximately equimolar concentration of IVTT protein. An IVTT reaction which used pET24d as the vector was added to normalize the total amount of IVTT mixture in each tube. The tubes were incubated for at least one hour at 4°C on a rocker. The beads were washed three times with the Binding

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Buffer and resuspended in 50 microliters of SDS loading buffer, which consisted of 0.25 Tris, pH 6.8, 5% (w/v) sucrose, 2% (w/v) SDS, 5% (v/v) 2-mercaptoethanol, and 0.005% (w/v) bromophenol blue. Samples were resolved by SDS-PAGE, and imaged using the Molecular Dynamics PhosphorImager (Sunnyvale, CA).

5   It is recognized that the GST-IVTT interaction assay system is not quantitative, and may depend on the relative association constant (k_{assoc}) which is related to the concentration of interacting peptides. Thus, subtle changes in the relative peptide concentrations may obscure potentially altered interactions. In order to provide control for such concentration-dependent processes between experiments, the molar
10   concentration of the GST-fusion protein and the molar concentration of the IVTT sample were determined. Furthermore, clear changes in interaction between hMLH1 and hPMS2 were observed by the inventors using a similar assay system that correlates with alterations known to be mutations, rather than polymorphisms.

           The results of the experiments presented in this Example are now
15   described.

GST Interaction Assay

           As described elsewhere herein, a physical interaction may be demonstrated between hMSH2 and either of hMSH3 and hMSH6 using immunoprecipitation (IP) reactions with anti-hMSH2 antibodies, which have been
20   described in the art and are publicly available. However, interaction-region mapping experiments using truncation mutants of hMSH3 and hMSH6 resulted in elevated background as a result of anti-hMSH2 antibody binding to the truncated probes. In addition, this IP assay did not appear sensitive enough to detect weak interactions.

           For these reasons, the alternative assay described herein was developed.
25   This assay relies on the use of a GST-fusion protein expressed in *E. coli* as a "bait" and *in vitro* transcribed and translated (IVTT) protein as "prey". This assay proved to be effective for all of the GST-fusion MutS homolog probe combinations used in the studies described in this application. These GST-fusion MutS homolog probe combinations included GST-hMSH2:IVTT-hMSH3, GST-hMSH3:IVTT-hMSH2,

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GST-hMSH2:IVTT-hMSH6, and GST-hMSH6:IVTT-hMSH2. The interaction for each of these IVTT full-length peptides was specific for the corresponding GST-hMSH(X) fusion protein, as evidenced by the observation that nearly undetectable background non-specific binding was demonstrated by incubation and

5   centrifugal precipitation of the IVTT-MSH(Y) with:

        1) GST-agarose beads alone;

        2) E. coli lysate +GST-agarose beads; and

        3) pGEX (the GST moiety alone) +GST-agarose beads as controls.

Furthermore, densitometric comparison of the PAGE lanes containing only pGEX with

10   PAGE lanes containing GST-hMSH(X) demonstrated that the signal-to-background ratio in this assay approaches 100. These results suggested that this bait-prey system was sufficient to map the interaction regions of the hMSH2-hMSH3 and the hMSH2-hMSH6 heterodimers.

        In these studies, a clear interaction between MSH homologs could be

15   demonstrated by comparing association of GST alone and IVTT-MSH(Y) with association of GST-MSH(X) and IVTT-MSH(Y), where X and Y are independently 2, 3, or 6. Furthermore, this assay provided a qualitative measure of interaction efficiency, because each experiment contained a nearly identical molar ratio of GST-MSH(X) and IVTT-MSH(Y). In addition, the GST-hMSH3 and GST-hMSH6

20   fusion proteins were demonstrated to be active for mis-pair binding when they are combined with purified hMSH2. These results indicate that the structure of the hMSH3 and hMSH6 proteins is not substantially altered by fusion to GST.

Interaction Regions of hMSH2 and hMSH3

        The regions of hMSH3 which interact with hMSH2 were determined, as

25   illustrated in Figure 12. Truncated hMSH3 polypeptides were constructed such that the protein was represented by three overlapping polypeptides, as illustrated in Figure 12, polypeptides 2-4. It was determined that there are two separate regions of hMSH3 that interact with hMSH2. It was recognized that an amino-terminal region of hMSH3 and a carboxy-terminal region of hMSH3 are involved in interactions with hMSH2, as

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illustrated, for example, by the abilities of polypeptides 5 and 10 in Figure 12 to interact with GST-hMSH2. The amino-terminal region was determined to be located within the region of hMSH3 bounded by amino acid residues 126 and 250, as indicated by the abilities of polypeptides 6-9 in Figure 12 to interact with GST-hMSH2. Because

5   the level of IVTT expression was insufficient for polypeptides comprising fewer than one hundred amino acids, the carboxy-terminal region was mapped using an internal deletion strategy. Using this strategy, the carboxy-terminal interaction region was determined to be located within the region of hMSH3 bounded by amino acid residues 1050 and 1128, as indicated by the abilities of polypeptides 10-14 in Figure 12 to

10   interact with GST-hMSH2.

The locations of regions of hMSH2 which interact with hMSH3 were determined in a similar fashion. The regions of hMSH2 which interact with hMSH3 were determined, as illustrated in Figure 13. Truncated hMSH2 polypeptides were constructed such that the protein was represented by four overlapping polypeptides, as

15   illustrated in Figure 13, polypeptides 2-5. It was determined that hMSH2 comprises two regions which are involved in interaction with hMSH3, as indicated by the abilities of polypeptides 1-6 in Figure 13 to interact with GST-hMSH3. An amino-terminal region was determined to be located within the region of hMSH2 bounded by amino acid residues 378 and 625, as indicated by the abilities of polypeptides 7-10 in Figure

20   13 to interact with GST-hMSH3. The amino acid boundaries of the carboxy-terminal interaction region of hMSH2 were not resolved with precision, due to sub-optimal signal strength. Nonetheless, the data illustrated in Figure 13 indicate that the carboxy-terminal interaction region of hMSH2 may at least be localized in the region bounded by amino acid residues 751 and 934 (the carboxy terminus), as indicated by the abilities

25   of polypeptide 6 in Figure 13 to interact with GST-hMSH3.

Because there were two interaction regions between hMSH2 and hMSH3, a system was designed to determine the linear orientation of the two regions. GST fusion proteins comprising truncated hMSH3 polypeptides were constructed. A GST-hMSH3 fusion protein comprising hMSH3 amino acid residues 1-297 comprised

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the consensus amino-terminal interaction region. A GST-hMSH3 fusion protein comprising hMSH3 amino acid residues 1025-1128)comprised the consensus carboxy-terminal interaction region. These two fusion proteins were used as "bait" against a series of hMSH2 "prey" truncation mutants. We found that non-truncated

5   hMSH2 interacted with both the GST-hMSH3 fusion protein, as indicated by the ability of polypeptide 1 in Figure 14 to interact with both the GST-hMSH3 fusion protein comprising the consensus amino-terminal interaction region and the GST-hMSH3 fusion protein comprising the consensus carboxy-terminal interaction region. The GST-hMSH3 fusion protein comprising the consensus amino-terminal

10   interaction region interacted most strongly with amino acid residues 251-750 of hMSH2 protein, as indicated by the ability of polypeptide 4 in Figure 14 to interact with this GST-hMSH3 fusion protein. The GST-hMSH3 fusion protein comprising the consensus carboxy-terminal interaction region interacted most strongly with amino acid residues 751-934 of hMSH2 protein, as indicated by the ability of polypeptides 5, 6, 7,

15   and 8 in Figure 14 to interact with this GST-hMSH3 fusion protein.

These results indicate that the amino-terminal interaction region of hMSH3 normally interacts with the amino-terminal interaction region of hMSH2 and that the carboxy-region interaction region of hMSH3 normally interacts with the carboxyl region interaction region of hMSH2. Use of the GST-hMSH3 fusion protein

20   comprising the consensus carboxy-terminal interaction region permitted further resolution of the carboxy-terminal interaction region of hMSH2. It was determined that the carboxy-terminal interaction region of hMSH2 is bounded by amino acid residues 875 and 934, as indicated by the ability of polypeptide 8 in Figure 14 to interact with this GST-hMSH3 fusion protein.

25   Interaction Regions of hMSH2 and hMSH6

Using a similar strategy, the locations of the interaction regions of hMSH2 and hMSH6 were determined. It was recognized that an amino-terminal region of hMSH6 and a carboxy-terminal region of hMSH6 are involved in interactions with hMSH2, as illustrated, for example, by the abilities of polypeptides 1-6 in Figure 15 to

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interact with GST-hMSH2. The amino-terminal region was determined to be located within the region of hMSH6 bounded by amino acid residues 326 and 575, as indicated by the abilities of polypeptides 7-10 in Figure 15 to interact with GST-hMSH2. The carboxy-terminal region was determined to be located within the region of hMSH6

5   bounded by amino acid residues 953 and 1360, as indicated by the abilities of polypeptide 6 to interact with GST-hMSH2.

The regions of hMSH2 which interact with hMSH6 were determined, as illustrated in Figure 16. Truncated hMSH2 polypeptides were constructed such that the protein was represented by four overlapping polypeptides, as illustrated in Figure 16,

10   polypeptides 2-5. It was determined that hMSH2 comprises two regions which are involved in interaction with hMSH6, as indicated by the abilities of polypeptides 1-6 in Figure 15 to interact with GST-hMSH6. The amino-terminal region was determined to be located within the region of hMSH2 bounded by amino acid residues 378 and 625, as indicated by the abilities of polypeptides 7-10 in Figure 15 to interact with GST-

15   hMSH6. Using a GST fusion protein which contained a truncation mutant of hMSH6 comprising amino acid residues 1302-1360, it was determined that the carboxyl terminal interaction region of hMSH2 is located within the region of hMSH2 bounded by amino acid residues 875 and 934, as indicated by the ability of polypeptide 8 in Figure 17 to interact with this GST fusion protein. The ability of polypeptide 8 in

20   Figure 17 to interact with this GST fusion protein also indicates that the carboxy-terminal interaction region of hMSH6 is bounded by amino acid residues 1302 and 1360.

These results indicate that the same amino acid regions of hMSH2 are involved in the interactions between hMSH2 and hMSH3 and the interactions between

25   hMSH2 and hMSH6.

The linear orientation of the hMSH2-hMSH6 interaction regions was determined. Using IVTT amino-terminal and carboxy-terminal hMSH2 interaction regions and GST fusion proteins comprising the amino-terminal and carboxy-terminal interaction regions of hMSH6, it was determined that the amino-terminal interaction

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region of hMSH6 interacts with the amino-terminal interaction region of hMSH2, as

indicated by the ability of polypeptides 3-5 in Figure 17 to interact with the GST fusion

protein comprising the amino-terminal interaction region of hMSH6. It was further

determined that the carboxy-terminal interaction region of hMSH6 interacts with the

5   carboxy-terminal interaction region of hMSH2, as indicated by the ability of

polypeptides 5-8 in Figure 17 to interact with the GST fusion protein comprising the

carboxy-terminal interaction region of hMSH6. Thus, the linear orientation of the

interaction regions of the hMSH2:hMSH6 heterodimer is identical to that of the

hMSH2:hMSH3 heterodimer.

10   Interaction Regions of hMSH2 with Itself

hMSH2 homodimers bind mismatched duplex DNA (Acharya et al.,

1996, Proc. Natl. Acad. Sci. USA 93:13629-13634). Using a GST-hMSH2 fusion

protein comprising hMSH2 amino acid residues 751-934, it was determined that this

portion of hMSH2 (i.e. the carboxy-terminal interaction region) interacts with the

15   carboxy terminus of hMSH2. Thus, the hMSH2 homodimer exhibits the same

carboxy-terminal interaction pattern that was observed between hMSH2 and either of

hMSH3 and hMSH6.

The Effect of hMSH2 Mutations Observed in HNPCC Kindreds on

hMSH(X):hMSH(Y) Interaction

20   Several HNPCC-associated missense mutations have been described

which are located within one of the two interaction regions of hMSH2 identified

herein. Six of these HNPCC-associated mutations were constructed, and the effect of

the mutations on hMSH(X):hMSH(Y) interaction were investigated, wherein X and Y

are independently 2, 3, or 6. The six HNPCC-associated mutations were those

25   designated L390V, K393M, R524P, N596D, P622L, and T905R. These mutations are

described in the HNPCC database (Peltomalei et al., 1997).

Interaction experiments were performed using mutated hMSH2

fragments which comprised either only an amino-terminal interaction region or a

carboxy-terminal interaction region to eliminate any confusion that the presence of

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multiple interaction regions might generate. These hMSH2 IVTT mutant consensus
interaction regions were examined for interaction with GST fusion proteins which
comprised either full length hMSH3 or full length hMSH6. No difference could be
discerned between binding of any mutated hMSH2 fragment to either of the fusion
5   proteins and binding of a corresponding wild type hMSH2 fragment to either of the
fusion proteins. These results suggest that altered interaction between hMSH2 and
either hMSH3 or hMSH6 are not likely to be causative functional defects resulting in
HNPCC.

The results of the experiments described in this Example suggest a
10   model for regional interactions of hMSH2 with hMSH3 and with hMSH6. This model
is illustrated in Figure 18. The results described herein indicate that hMSH2 employs
the same interaction regions, regardless of whether it interacts with hMSH3 or with
hMSH6. These interactions are mediated by two distinct regions of hMSH2, an amino-
terminal interaction region bounded by amino acid residues 378 and 625 and a
15   carboxy-terminal interaction region bounded by amino acid residues 875 and 934. The
adenine nucleotide binding region and the putative helix-turn-helix motif of hMSH2
are not contained within either of these regions. Thus, the results described in this
Example indicate that it is unlikely that helix-turn-helix is essential for interaction of
hMSH2 with hMSH3 or with hMSH6. Figure 18 illustrates both the relative positions
20   and the linear orientation of the interaction regions of hMSH2, hMSH3, and hMSH6.

Since hMSH3 and hMSH6 appear to contact hMSH2 within the same
binding regions, the amino terminal and carboxyl terminal regions of hMSH3 and
hMSH6 were aligned and compared. The amino terminal interaction regions of
hMSH3 and hMSH6 exhibited little identifiable homology. The carboxyl terminal
25   interaction regions of hMSH3 and hMSH6 exhibited moderate homology, 16 of 60
residues being identical. The carboxyl-terminal regions of hMSH3 and hMSH6 may
provide a conserved function for these proteins such as, but not limited to,
protein-protein interaction.

Example 5

hMSH5. A Human MutS Homolog that Participates
in the Second Meiotic Division

In the experiments presented in this Example, the human MSH5 protein

5   (hMSH5) and the cDNA sequence encoding it are described. The *msh5* gene is located

at chromosome 6p22-21, and is involved in meiosis, as evidenced by expression of

msh5 in the testes and confinement of such expression to secondary spermatocytes and

developing spermatids. hMSH5 specifically interacts with hMSH4, confirming the

generality of functional heterodimeric interactions in eukaryotic MutS homologs. The

10   hMSH4:hMSH5 heterodimer may thus be analogized with the hMSH2:hMSH3 and

hMSH2:hMSH6 heterodimers.

The materials and methods described in the experiments presented in

this Example are now described.

Cloning the hMSH4 and hMSH5 cDNAs

15   A search of the NCBI EST database indicated that a 466-base pair

sequence derived from Soars human fetal liver spleen cDNA (T67203) exhibited

significant homology with both yeast MSH3 and yeast MSH5. The amino acid

sequence of the yeast and the human MSH2 homologs were used to screen the Human

Genome Sciences (HGS, Bethesda, MD) computer database using TFASTA computer

20   software designed by the Genetics computer Group (GCG, University of Wisconsin).

The HGS database contains nucleotide sequence information of expressed sequence

tags (ESTs) which identify a diverse collection of cDNAs derived from more than 400

cDNA libraries (Adams et al., 1991, Science 252:1651-1656). One EST (designated

C4) was determined to exhibit significant homology, but not identity, to yeast and

25   human MSH2 and MSH3 protein sequences.

Two PCR fragments were amplified using primers derived from these

two EST sequences, which were identified in cDNA derived from human testis. The

PCR product were used to screen a normal human testis cDNA library (obtained from

Clontech, Palo Alto, CA) using conventional plaque hybridization techniques. One of

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the primer sets derived from C4 yielded a consistent sequence and identified numerous phage clones. This set of primers comprised a forward primer (5'-ACGCCATCTT CACACGAAT-3'; SEQ ID NO: 31) and a reverse primer (5'-TGCAGTGGCA TTGTTCACT-3'; SEQ ID NO: 32). Six clones were identified which were amplified

5   using these primers, and these clones were excised using the pDR2 phagemid, according to the manufacturer's recommendations. The six clones were subcloned into pBSK (Stratagene, La Jolla, CA), and double strand sequencing of the six clones was performed using the PRISM™ Ready Reaction DyeDeoxy Terminator Cycle Sequencing Kit and an Applied Biosystems 377 Sequencer (Foster City, CA).

10   One clone, designated b29, comprised an open reading frame (ORF) 2505 base pairs in length. This ORF comprised one STOP codon N-terminal to the start methionine codon and one STOP codon at a position corresponding to the C-terminus of the protein encoded by the ORF. The completeness of the N-terminal region of the ORF was confirmed by performing a RACE reaction using human normal

15   testis cDNA (Clontech, Palo Alto, CA), as described (Apte et al., 1993, BioTechniques 15:890-893). The EST sequence obtained from NCBI (T67203) was found to be located in the C-terminal portion of the b29 ORF.

Clone b29 was further subcloned into pGEX (Pharmacia, Piscataway, NJ) for expression of the GST fusion protein in *E. coli* XL1 Blue (Stratagene, La Jolla,

20   CA) and into pET29a (Clontech, Palo Alto, CA) for *in vitro* transcription and translation (IVTT) using restriction endonucleases *Nde*I and *Not*I (New England Biolabs, Beverley, MA).

An hMSH4 clone was obtained from human testis cDNA (Clontech, Palo Alto, CA) by PCR amplification and subsequent ligation into the pCR2.1 vector

25   using a TA cloning kit (Invitrogen, San Diego, CA). The primer sequences which were used in these reactions included an outer forward primer (5'-GGAAGGTTTG GGAGGATGC TGAGG-3'; SEQ ID NO: 33), a reverse primer (5'-ATTGTGATTA TTCTTCAGTC TT-3'; SEQ ID NO: 34), a nested PCR: forward primer (5'-ATCTCGAGAT GCTGAGGCCT GAG-3'; SEQ ID NO: 35), and a second reverse

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primer (5'-GCGCTAGCTT ATTCTTCAGT CTTTTC-3'; SEQ ID NO: 36). The
nucleotide sequence of the amplified clone was confirmed by complete double strand
sequencing of both strands.

5

The hMSH4 clone contained a deletion of a C residue in codon 18 and
an insertion of a G residue in codon 20, resulting in V19S and V20S mutations.
Furthermore, the hMSH4 clone contained a G→A mutation at base 1219 of the
published sequence (numbered starting with the A in the ATG initiator codon), which
resulted in an E407K amino acid substitution. In addition, an apparent polymorphism
at codon 368 (CGC→AGA) was detected, which does not alter the coding Arg.

10
Chromosomal Mapping of hMSH5

PCR reactions were performed using the primers described above
respectively, to screen the Genebridge-4™ Radiation Hybrid Panel (Hudson et al.,
1995, Science 270:1945-1954). 35 amplification cycles were performed using an
annealing temperature of 60°C for 30 seconds followed by 72°C for 1 minute.

15
Fragments were visualized by agarose gel electrophoresis.

Northern Blotting

Three multiple tissue northern blots containing poly-A + RNA obtained
from a total of 23 different human tissues were obtained from Clontech (Palo Alto,
CA). 50 nanograms of a full length hMSH5 cDNA and a beta-actin cDNA control were

20
radiolabeled using alpha-(^{32}P)-dCTP by random primed labeling (Boehringer
Mannheim, Germany). Northern Blots were hybridized according to the manufacturer's
instructions. The blots were washed in 2 × SSC containing 0.05% (w/v) SDS at room
temperature (i.e. about 20°C) for a total of 60 minutes and at 50°C in 0.1 × SSC, 0.1 %
(w/v) SDS for a total of 40 minutes. Phosphorimager screens were exposed for one

25
day. A 2.5-2.6 kilobase transcript was detected at a high level in testis. Tissues with
significantly lower expression levels included bone marrow, lymph nodes, brain, and
spinal cord.

Antibodies

Five different 15-mer peptides were synthesized, each corresponding to predicted immunogenic regions of the hMSH5 protein. These peptides were conjugated to hemocyanin, and polyclonal antibodies were raised in rabbits (H.T.I.

5   Bio-Products, Ramona, CA). Antibody clone C924-2 was found to be most sensitive and specific in Western Blot experiments and was purified over a Protein-A column for Western analysis. Further affinity purification of the antibody was performed using a crude lysate of SF9 insect cells overexpressing hMSH5 protein. hMSH5 protein lysate was separated by SDS-PAGE, transferred to nitrocellulose and the hMSH5 specific

10   region excised and used to affinity purify the antibody as described (Wilson et al., 1995, Cancer Res. 55:5146-5150).

Immunohistochemistry

5-micron sections of formalin-fixed and paraffin embedded tissues were cut onto Neoprene coated slides (Aldrich Chemicals, Milwaukee, WI). After de-

15   paraffinization, including a 30 minute methanolic peroxide block for endogenous peroxidase activity (Leica Autostainer, Leica, Deerfield, IL), the slides were subjected to microwave radiation in 200 milliliters of Chem.Mate H.I.E.R buffer, pH 5.5-5.7 (Ventana Medical Systems, Tucson, AZ) at high energy for 5 minutes using a Panasonic Microwave #NN-5602A (Franklin PK, IL). 50 milliliters of water were

20   replaced for additional microwave exposure for 4 minutes at high energy .

Immunostaining using the catalyzed signal amplification system (DAKO™, Carpinteria, CA) was performed according to the manufacturer's instructions. Incubation with Protein-A and hMSH5 specific affinity purified polyclonal antibody was performed for 50 minutes at room temperature at

25   concentrations of 1:800 or 1:2000, respectively, using the hMSH2 polyclonal antibody. For counter staining with Harris Hematoxylin (Surgipath, Richmond, IL), the Leica Autostainer was used.

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GST Fusion Protein Interaction assay

500 microliters taken from a 5 milliliter overnight starter culture of cells which expressed an hMSH2-, hMSH3-, hMSH5-, or hMSH6-pGEX-fusion protein with (or non-fused pGEX as a negative control) was inoculated into 50 milliliters of

5   Luria broth which contained 50 micrograms per milliliter ampicillin, and this culture was grown until the optical density at 600 nanometers was about 0.5. Protein expression was induced by addition of 0.1 millimolar (final concentration) IPTG for 2 hours at 30°C. Cells were pelleted and resuspended in 750 microliters of phosphate buffered saline containing protease inhibitors. A 10 minute digestion on ice using 1

10   milligram per milliliter lysozyme was then performed. After the addition of 0.2% (v/v) Triton X- 100 and 1 millimolar dithiothreitol (final concentrations), the lysate was snap-frozen in liquid nitrogen and thawed twice. DNaseI (200 units per milliliter; Boehringer Mannheim, Germany) digestion was performed using the thawed lysate for 30 minutes on ice, after which cell debris was removed by centrifugation at 14,000 rpm

15   at 4°C for 30 minutes in a benchtop microfuge. Equal amounts of lysates obtained from cultures which separately expressed one of the fusion proteins (or GST alone as a negative control) were incubated on a rocking platform for 1 hour at 4°C in the presence of 2 milligrams of glutathione-agarose beads (Sigma Chemical Co., St. Louis, MO) which had been pre-swollen in phosphate buffered saline containing protease

20   inhibitors for 1 hour at room temperature. The beads were washed three times with 500 microliters of Interaction Buffer, which comprised 20 millimolar Tris-HCl, pH 7.5, 10% (v/v) Glycerol, 150 millimolar NaCl, 0.1% (v/v) Tween 20, 5 millimolar EDTA, I millimolar DTT, 0.75 milligrams per milliliter bovine serum albumin (Amresco, Solon, OH), and proteinase inhibitors). The beads were subsequently incubated in Interaction

25   buffer for 1 hour at 4°C on a rocking platform.

In vitro transcriptions and translation (IVTT) reactions were performed using 1 microgram each of hMSH2, hMSH3, hMSH5, and hMSH6 inserts (separately) in pET vectors and using the hMSH4 insert in pCR 2.1 using the TNT coupled reticulocyte lysate system (Promega, Madison, WI) according to the manufacturer's

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protocol. About 40 microcuries of ^{35}S-methionine was incorporated into each protein. 5 microliters of individual IVTTs was added to 500 microliters of glutathione-agarose beads in Interaction buffer, and the mixture was incubated for 1 hour at 4°C on the rocking platform. After three final washing steps, the supernatant was removed, and

5   the beads were resuspended in 35 microliters of 2 × Spear's buffer, boiled for 5 minutes, and centrifuged for 5 minutes at 14,000 rpm in a benchtop microfuge. 15 microliters of each reaction mixture was loaded onto separate lanes of an 8% (w/v) SDS-PAGE Gel (BioRad MiniProtean II), and electrophoresis was performed for about 90 minutes at 135 volts. PhosphorImager screens (Molecular Dynamics) were exposed

10   to the dried gels for one day.

The results of the experiments described in this Example are now described:

Isolation an chromosomal map of hMSH5, a new human MutS homolog

Six clones which contained the EST later determined to correspond to

15   msh5 were isolated, and the nucleotide sequence of both strands of the clone inserts were determined. Sequence analysis of clone b29 indicated the presence of an ORF 2505 base pairs in length. This ORF encoded putative 834-amino-acid protein, as indicated in Figures 19A-19C. The predicted molecular weight of the protein is 97 kilodaltons. A STOP codon was identified beyond the N-terminal end of the ORF, in

20   the non-coding region, and the completeness of the ORF was confirmed by T-RACE analysis.

The Genebridge-4 Radiation Hybrid Panel for PCR products having a length corresponding to this ORF. In this way, the msh5 gene was located 6.94cR from D6S478 on chromosome 6p22.1-21.3.

25   MSH5 defines a new family of MutS homologs involved in sporulation and meiosis

Of all eukaryotic and prokaryotic MutS homologs, the b29 clone was found to be most closely related to Caenorhabdis elegans MSH5 (29% amino acid identity) and Saccharomyces cerevisiae MSH5 (25% amino acid identity). A region encompassing the adenine nucleotide binding domain displayed approximately 60%

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amino acid identity among these homologues. The gene was therefore designated human *msh5*.

Among MutS homologs, the next closest relatives to hMSH5 are the MSH2 proteins. hMSH3 and hMSH6 proteins appear to be less closely related to hMSH5 than are the bacterial MutS proteins. In the present alignment, the MSH4 proteins appear to be the most divergent of the MutS homologs.

Expression of hMSH5

Human *msh5* was determined to be transcribed at a high level in testis (Figure 3). These results correspond to the observation that, in yeast, MSH5 expression was meiosis specific (Hollingsworth et al., 1995, Genes Dev. 9:1728-1739). The size of the human transcript corresponded to the length of the cDNA sequence, which is 2.5 kilobases. The presence of hMSH5 was detected in testis and tonsil tissue and, at very low levels, in two T- and B-cell tumor lines (Jurkat, CEM, Daudi, and GM 1500 cell lines) by Western Blot analysis. The Western signal in these autopsy tissues revealed low molecular weight protein band(s) that were likely degradation products of the significant autolytic reactions occurring in these samples. *msh5* expression was also observed in human bone marrow and lymph node tissues. The presence of *msh5* transcript in human tissues where B- and T-cells develop as well as expression in the T- and B-cell lines suggests a relationship to cellular development processes that include recombination events. However, it is also possible that the low levels of hMSH5 protein expression in the B- and T-cell lines could result from the fact that the cell lines are derived from hematologic malignancies and thus do not represent normal B- and T-cell precursors or other undefined factors. hMSH5 expression may also occur in human brain, spinal cord, and trachea tissues.

Western analysis suggested that several of the purified polyclonal antibodies derived from synthetic peptides are useful use immunohistochemical (IHC) studies. IHC stains for surgical specimens obtained from patients with various testicular tumors exhibited nuclear expression of hMSH5 in spermatids *in statu nascendi* in round and elongated spermatids (S3). In contrast, all of the preceding

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phases of spermatogenesis, as well as the spermatozoa themselves exhibited no expression of hMSH5. These observations indicate that hMSH5 has a specific role in the processes associated with the second meiotic division.

The testicular histology of the orchiectomy specimens was not entirely normal. Thus, it is possible that hMSH5 was abnormally expressed in the testicular samples obtained from surgical patients. In the samples examined, histological examination revealed occasional intratubular neoplasia and the presence of discrete lymphocytic infiltrates. However, spermatogenesis in these samples was still functioning sufficiently to produce mature sperm cells and a number of tubules were found where there was no evidence of neoplasia. Furthermore, staining of spermatids was evident in all of the tubules that appeared normal based on the presence of all stages of spermatogenesis. Textbook examples of normal tubules would show the cell types of spermatogenesis filling the entire tubule.

In contrast, hMSH2 is expressed in the nuclei at nearly all phases of spermatogenesis except for the round and elongated spermatids (where hMSH5 is expressed) and the spermatozoa. Sertoli cells exhibit faint nuclear staining with hMSH2-specific antibody. hMSH2 expression in tissue is clearly correlated with proliferation in general, which is exemplified in the experiments described in this Example by nuclear expression of hMSH2 in the seminoma. In addition, tissues that were positive for hMSH2 expression were also positive for expression of the proliferation marker Ki67. hMSH5 protein expression was absent in seminoma and other testicular malignancies such as embryonal cell carcinoma and mature and immature teratoma. Expression of hMSH5 was absent in dividing spermatogonium A, suggesting that expression is not induced during mitosis.

Protein Interaction Studies

Because hMSH2, hMSH3 and hMSH6 are, as described herein, known to act as heterodimers, interaction studies of hMSH5 with hMSH2, hMSH3, hMSH4 and hMSH6 were performed.

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hMSH2 interacts strongly with hMSH3 and hMSH6, as described herein in Example 1. IVTT-hMSH5 did not interact with GST-hMSH2, -hMSH3 or hMSH6 fusion proteins. Similarly, none of IVTF-hMSH2, -hMSH3, and -hMSH6 interacted with GST-hMSH5. The lack of interaction of hMSH5 with hMSH2, hMSH3, and

5   hMSH6 was confirmed as the intensity of the bands never exceeded the background. However, there was significant interaction of GST-hMSH5 with IVTT-hMSH4. Furthermore, a significant interaction of GST-hMSH3 fusion protein with IVTT-hMSH4 was observed. However, this potential interaction could not be confirmed since significant amounts of soluble GST-hMSH4 fusion protein could not be obtained.

10   These results suggest that hMSH5 specifically interacts with hMSH4 alone.

In yeast, *msh5* mutants have decreased spore viability, increased levels of Meiosis I chromosomal nondisjunction and decreased levels of reciprocal exchange between, but not within, chromosomes (Hollingsworth et al., 1995, Genes Dev. 9:1728-1739). This observation, combined with the results described herein suggest that

15   hMSH5, and thus also hMSH4, is involved in meiotic processing. hMSH5 is located on chromosome 6p22-21 and is expressed at very high levels in the testis where meiosis occurs continually throughout adult life. Immunohistochemical examination of testicular sections revealed that the protein expression of hMSH5 occurred in developing round and elongated spermatids. Spermatogonia and primary

20   spermatocytes did not express hMSH5, and expression of hMSH5 ended abruptly upon development of mature sperm. Because the expression of hMSH5 is exceedingly strong in the round spermatocytes, it is likely that expression of hMSH5 begins in the secondary spermatocyte. The expression pattern of hMSH5 is consistent with the phenotypes exhibited in yeast, since the meiosis I chromosomal non-disjunction occurs

25   at the cellular division between the primary and secondary spermatocyte, at the stage where the expression of hMSH5 is likely to be initiated.

The observations described herein that hMSH5 was expressed in human tissues such as bone marrow and lymph nodes, where T-cell and B-cell development

takes place, suggests that hMSH5 has a role in development of B-cells, T-cells, or both, and that defects in hMSH5 might result in hematological defects.

hMSH5 appears to specifically interact with hMSH4. No interaction with hMSH5 above background was observed for hMSH2, hMSH3 or hMSH6. Thus,

5   it is likely that the hMSH4-hMSH5 heterodimer is specific and constitutes a functional interaction that is separate from hMSH2-hMSH3 and hMSH2-hMSH6 heterodimers. Based on the conservation of the adenine nucleotide binding and hydrolysis domain, it is likely that the hMSH4-hMSH5 heterodimer also functions as a molecular switch (Gradia et al., 1997, Cell 91:995-1005).

10   The disclosures of each and every patent, patent application, and publication cited herein are hereby incorporated herein by reference in their entirety.

While this invention has been disclosed with reference to specific embodiments, it is apparent that other embodiments and variations of this invention may be devised by others skilled in the art without departing from the true spirit and

15   scope of the invention. The appended claims are intended to be construed to include all such embodiments and equivalent variations.

What is claimed is:

1. A method of modifying a mismatched duplex DNA, said method comprising contacting an MSH dimer and said mismatched duplex DNA in the presence of a binding solution comprising a nucleotide selected from the group consisting of ADP and ATP, wherein the concentration of ATP in said binding solution is less than about 3 micromolar, whereby said MSH dimer associates with the mismatched region of said mismatched duplex DNA, thereby modifying said mismatched duplex DNA.

2. The method of claim 1, wherein said MSH dimer is selected from the group consisting of a prokaryotic MSH homodimer, a prokaryotic MSH heterodimer, a eukaryotic MSH homodimer, and a eukaryotic MSH heterodimer.

3. The method of claim 2, wherein said MSH dimer is a homodimer of a MutS homolog selected from the group consisting of a human MutS homolog, a murine MutS homolog, a rat MutS homolog, a *Drosophila* MutS homolog, a yeast MutS homolog, and a *Saccharomyces cerevisiae* MutS homolog.

4. The method of claim 2, wherein said eukaryotic MSH homodimer is an MSH2 homodimer.

5. The method of claim 2, wherein said eukaryotic MSH heterodimer comprises MutS homologs independently selected from the group consisting of an MSH2 protein, an MSH3 protein, an MSH4 protein, an MSH5 protein, and an MSH6 protein.

6. The method of claim 5, wherein said MSH dimer is selected from the group consisting of an MSH2:MSH3 heterodimer, an MSH2:MSH6 heterodimer, and an MSH4:MSH5 heterodimer.

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7. The method of claim 2, wherein said prokaryotic MSH dimer is a homodimer of *Escherichia coli* MutS.

8. The method of claim 1, wherein said MSH dimer is substantially purified.

5

9. The method of claim 1, wherein the concentration of ATP in said binding solution is less than about 0.3 micromolar.

10. The method of claim 9, wherein said binding solution is substantially free of ATP.

11. The method of claim 1, wherein at least one of said MSH dimer and

10 said mismatched duplex DNA is bound to a support.

12. The method of claim 1, wherein said mismatched duplex DNA has at least one free end.

13. The method of claim 1, wherein said mismatched duplex DNA comprises a DNA strand generated by reverse transcription of mRNA obtained from an

15 organism.

14. The method of claim 1, wherein said mismatched duplex DNA comprises a first DNA strand having a reference nucleotide sequence and a second DNA strand selected from the group consisting of a DNA strand obtained from an organism, a DNA strand obtained by amplification of at least a portion of a

20 polynucleotide obtained from an organism, a DNA strand obtained by cleavage of a polynucleotide obtained from an organism, and a DNA strand obtained by reverse transcription of a polynucleotide obtained from an organism.

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15. The method of claim 14, wherein said second DNA strand comprises at least a portion of a gene associated with a cancer in said organism.

16. The method of claim 15, wherein said organism is a human and wherein said gene is selected from the group consisting of an oncogene and a tumor

5   suppressor gene.

17. The method of claim 16, wherein said gene is selected from the group consisting of *abl, akt2, apc, bcl2α, bcl2β, bcl3, bcr, brca1, brca2, cbl, ccnd1, cdk4, crk-II, csf1r/fms, dbl, dcc, dpc4/smad4, e-cad, e2f1/rbap, egfr/erbb-1, elk1, elk3, eph, erg, ets1, ets2, fer, fgr/src2, fli1/ergb2, fos, fps/fes, fra1, fra2, fyn, hck, hek,*

10   *her2/erbb-2/neu, her3/erbb-3, her4/erbb-4, hras1, hst2, hstf1, ink4a, ink4b, int2/fgf3, jun, junb, jund, kip2, kit, kras2a, kras2b, lck, lyn, mas, max, mcc, met, mlh1, mos, msh2, msh3, msh6, myb, myba, mybb, myc, mycl1, mycn, nf1, nf2, nras, p53, pdgfb, pim1, pms1, pms2, ptc, pten, raf1, rb1, rel, ret, ros1, ski, src1, tal1, tgfbr2, thra1, thrb, tiam1, trk, vav, vhl, waf1, wnt1, wnt2, wt1,* and *yes1.*

15   18. The method of claim 17, wherein said cancer is hereditary non-polyposis colon cancer and said gene is selected from the group consisting of *mlh1, msh2, msh3, msh6, pms1,* and *pms2.*

19. The method of claim 15, wherein said cancer is selected from the group consisting of a leukemia, a lymphoma, a meningioma, a mixed tumor of a

20   salivary gland, an adenoma, a carcinoma, an adenocarcinoma, a sarcoma, a dysgerminoma, a retinoblastoma, a Wilms' tumor, a neuroblastoma, a melanoma, and a mesothelioma.

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20. The method of claim 1, wherein said mismatched duplex DNA and said MSH dimer are contacted in the presence of at least one non-mismatched duplex DNA.

21. The method of claim 20, further comprising separating said MSH dimer from said non-mismatched duplex DNA after contacting said mismatched duplex DNA and said MSH dimer.

22. The method of claim 21, further comprising dissociating said mismatched duplex DNA and said MSH dimer after separating said MSH dimer from said non-mismatched duplex DNA and thereafter amplifying said mismatched duplex DNA.

23. The method of claim 22, wherein said MSH dimer is bound to a support prior to separating said non-mismatched duplex DNA from said MSH dimer.

24. The method of claim 23, wherein said non-mismatched duplex DNA is separated from said MSH dimer in the presence of a separating solution, wherein said separating solution is substantially free of ATP.

25. The method of claim 24, further comprising releasing said mismatched duplex DNA from said MSH dimer after separating said non-mismatched duplex DNA from said MSH dimer.

26. The method of claim 25, wherein said mismatched duplex DNA has at least one free end and is released from said MSH dimer by contacting said MSH dimer with a releasing solution selected from the group consisting of a solution comprising ATP and Mg^{2+} ions, a solution comprising ATP and a magnesium-chelating agent, a solution comprising high salt, a solution comprising a gamma-

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modified ATP analog and Mg^{2+} ions, and a solution comprising a gamma-hydrolysis-resistant ATP analog and Mg^{2+} ions.

27. The method of claim 26, wherein said releasing solution comprises ATP and Mg^{2+} ions.

5   28. The method of claim 25, wherein said mismatched duplex DNA does not have a free end and is released from said MSH dimer by contacting said MSH dimer with a releasing solution selected from the group consisting of a solution comprising a magnesium-chelating agent, a solution comprising high salt, a solution comprising a double-stranded DNA cleaving enzyme, ATP and Mg^{2+} ions, a solution

10   comprising a double-stranded DNA cleaving enzyme, a gamma-modified ATP analog, and Mg^{2+} ions, and a solution comprising a double-stranded DNA cleaving enzyme, a gamma-hydrolysis-resistant ATP analog, and Mg^{2+} ions.

29. The method of claim 21, further comprising contacting said MSH dimer with a MutL homolog after contacting said mismatched DNA and said MSH

15   dimer.

30. The method of claim 1, further comprising detecting association of said MSH dimer with said mismatched duplex DNA.

31. The method of claim 30, wherein association of said MSH dimer with said mismatched duplex DNA is detected using an assay selected from the group

20   consisting of a gel mobility shift assay, a filter binding assay, an immunological assay, a sedimentation centrifugation assay, a spectroscopic assay, an optical affinity assay, a DNA footprint assay, and a nucleolytic cleavage protection assay.

32. The method of claim 1, wherein said duplex DNA does not have a free end.

33. The method of claim 32, wherein said MSH dimer is present in molar excess with respect to said mismatched duplex DNA, whereby an average of
5   more than one said MSH dimer associates with one molecule of said mismatched duplex DNA.

34. A method of modifying a mismatched duplex DNA which does not have a free end, said method comprising contacting said mismatched duplex DNA and an MSH dimer having ADP bound thereto in the presence of a binding solution,
10   wherein the concentration of ATP in said binding solution is less than about 3 micromolar, whereby said homolog associates with the mismatched region of said mismatched duplex DNA, thereby modifying said mismatched duplex DNA.

35. A method of segregating a mismatched duplex DNA from a population of DNA molecules, said method comprising
15   contacting an MSH dimer and said population in the presence of a binding solution comprising a nucleotide selected from the group consisting of ADP and ATP, wherein the concentration of ATP in said binding solution is less than about 3 micromolar, whereby said MSH dimer associates with said duplex DNA; and
        segregating said MSH dimer from said population, whereby said mismatched
20   duplex DNA is segregated from said population.

36. A method of detecting a difference between a sample nucleotide sequence and a reference nucleotide sequence, said method comprising
a)   annealing a first DNA strand and a second DNA strand to form a duplex DNA,
        i)   wherein said first DNA strand has said sample nucleotide sequence

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ii)   wherein said second DNA strand has a nucleotide sequence which is

complementary to said reference nucleotide sequence, and

iii)   whereby if there is a difference between said sample nucleotide

sequence and said reference nucleotide sequence then said duplex DNA is a

5   mismatched duplex DNA;

b)   thereafter contacting said duplex DNA and an MSH dimer in the presence of a

binding solution comprising a nucleotide selected from the group consisting of ADP

and ATP, wherein the concentration of ATP in said binding solution is less than about

3 micromolar, whereby said MSH dimer associates with said duplex DNA if said

10   duplex DNA is a mismatched duplex DNA; and

c)   determining whether said MSH dimer is associated with said duplex DNA

molecule, whereby association of said MSH dimer with said duplex DNA molecule is

an indication that there is a difference between said sample nucleotide sequence and

said reference nucleotide sequence.

15   37. A kit for separating a mismatched duplex DNA from non-

mismatched duplex DNAs, said kit comprising

at least two MutS homologs;

a linker for binding said at least one of said MutS homologs to a support; and

an additional reagent selected from the group consisting of a nucleotide and a

20   releasing solution, wherein said nucleotide is selected from the group consisting of

ADP and ATP, and wherein said releasing solution comprises Mg^{2+} and a compound

selected from the group consisting of ATP, a gamma-modified ATP analog, and a

gamma-hydrolysis-resistant ATP analog.

                 38. A method of determining whether a mammal is predisposed for

25   carcinogenesis, said method comprising

a)   annealing a first DNA strand and a second DNA strand to form a duplex DNA,

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i)   wherein said first DNA strand has the nucleotide sequence of at least a

portion of a gene selected from the group consisting of an oncogene and a

tumor suppressor gene of said mammal, and

ii)   wherein said second DNA strand has a nucleotide sequence which is

5   complementary to the consensus nucleotide sequence of said region,

iii)   whereby if there is a sequence difference between said first DNA strand

and said second DNA strand then said duplex DNA is a mismatched duplex

DNA;

b)   thereafter contacting said duplex DNA and an MSH dimer in the presence of a

10   binding solution comprising a nucleotide selected from the group consisting of ADP

and ATP, wherein the concentration of ATP in said binding solution is less than about

3 micromolar, whereby said MSH dimer associates with said duplex DNA if said

duplex DNA is a mismatched duplex DNA; and

c)   determining whether said MSH dimer is associated with said duplex DNA,

15   whereby association of said MSH dimer with said duplex DNA is an indication that

said mammal is predisposed for carcinogenesis.

39.   A method of fractionating a population of duplex DNAs, said

method comprising

a)   contacting said population with an MSH dimer in the presence of a binding

20   solution comprising a nucleotide selected from the group consisting of ADP and ATP,

wherein the concentration of ATP in said binding solution is less than about 3

micromolar, whereby said MSH dimer associates with at least one mismatched duplex

DNA in said population; and

b)   segregating said MSH dimer from said population of duplex DNAs, whereby

25   said mismatched duplex DNA is segregated from said population of duplex DNAs,

whereby said population is fractionated.

40. A method of selectively amplifying at least one mismatched duplex DNA of a population of duplex DNAs, said method comprising

contacting said population with an MSH dimer in the presence of a binding solution comprising a nucleotide selected from the group consisting of ADP and ATP,

5   wherein the concentration of ATP in said binding solution is less than about 3 micromolar, whereby said MSH dimer associates with said mismatched duplex DNA,

thereafter segregating said MSH dimer from said population of duplex DNAs, whereby said mismatched duplex DNA is segregated from said population of duplex DNAs, and

10   thereafter amplifying said mismatched duplex DNA, whereby said mismatched duplex DNA is selectively amplified.

41. A method of determining whether the nucleotide sequence of a first copy of a genomic sequence differs from the nucleotide sequence of a second copy of said genomic sequence, said method comprising

15   amplifying a region of each of said first copy and said second copy of said genomic sequence to yield amplified first copies and amplified second copies;

mixing and denaturing said amplified first copies and said amplified second copies to form a first mixture;

thereafter annealing the nucleic acids in said first mixture to form a second

20   mixture comprising duplex DNAs, whereby if said the nucleotide sequence of first copy and the nucleotide sequence of said second copy of said genomic sequence differ then at least some of said duplex DNAs are mismatched duplex DNAs;

thereafter contacting said second mixture with an MSH dimer in the presence of a binding solution comprising a nucleotide selected from the group consisting of ADP

25   and ATP, wherein the concentration of ATP in said binding solution is less than about 3 micromolar, whereby said MSH dimer associates with said mismatched duplex DNAs; and

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determining whether said MSH dimer is associated with at least some of said duplex DNAs, whereby association of said MSH dimer with said at least some of said duplex DNAs is an indication that the nucleotide sequence of said first copy of said genomic sequence differs from the nucleotide sequence of said second copy of said

5   genomic sequence.

42. A composition for segregating a mismatched duplex DNA from a population of duplex DNAs, said composition comprising an MSH heterodimer bound to a support.

43. A kit for screening a genomic region for a nucleotide sequence

10   which differs from a reference nucleotide sequence, said kit comprising
a pair of primers complementary to the ends of said region for amplifying said region;
a DNA strand having said reference nucleotide sequence; and
at least two MutS homologs.

15   44. A nonhuman mammal which is nullizygous for both *Msh2* and *p53*, wherein said mammal does not express *Msh2* or *p53*, and wherein said mammal exhibits a phenotype selected from the group consisting of inappropriate fetal apoptosis and a predisposition for carcinogenesis.

45. A method of making a nonhuman mammal which is nullizygous for

20   both *Msh2* and *p53*, does not express *Msh2* or *p53*, and exhibits a phenotype selected from the group consisting of a predisposition for inappropriate fetal apoptosis and a predisposition for carcinogenesis, said method comprising mating
a)   a first parent mammal comprising at least one null allele of *Msh2* and at least one null allele of *p53* and

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b)   a second parent mammal comprising at least one null allele of *Msh2* and at least one null allele of *p53*,

whereby a nonhuman mammal is generated which is nullizygous for both *Msh2* and *p53*, does not express *Msh2* or *p53*, and exhibits a phenotype selected from the group consisting of inappropriate fetal apoptosis and a predisposition for carcinogenesis.

46. A method of determining whether a compound affects tumorigenesis in mammals, said method comprising

administering said compound to a first nonhuman mammal which is nullizygous for both *Msh2* and *p53*, does not express *Msh2* or *p53*, and exhibits a predisposition for carcinogenesis, and

comparing tumor incidence in said first nonhuman mammal with tumor incidence in a second nonhuman mammal of the same type which is nullizygous for both *Msh2* and *p53*, does not express *Msh2* or *p53*, exhibits a predisposition for carcinogenesis, and to which said compound is not administered,

whereby a difference in tumor incidence in said first transgenic mammal compared with tumor incidence in said second transgenic mammal is an indication that said compound affects tumorigenesis in mammals.

47. A method of determining whether a compound affects a biological phenomenon in mammals, said phenomenon selected from the group consisting of apoptosis, aging, and fetal development, said method comprising

administering said compound *in utero* to a first nonhuman mammalian embryo which is nullizygous for both *Msh2* and *p53*, does not express *Msh2* or *p53*, and exhibits a predisposition for inappropriate fetal apoptosis, and

comparing the development of said first nonhuman mammalian embryo with the development of a second nonhuman mammalian embryo of the same type which is nullizygous for both *Msh2* and *p53*, does not express *Msh2* or *p53*, exhibits a

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predisposition for inappropriate fetal apoptosis, and to which said compound is not
administered,

 whereby a difference in the development of said first nonhuman mammalian
embryo compared with the development of said second nonhuman mammalian embryo
5 is an indication that said compound affects said biological phenomenon in mammals.

   48. A cell line which is nullizygous for both *Msh2* and *p53*, does not
express *Msh2* or *p53*, and exhibits a phenotype selected from the group consisting of a
predisposition for carcinogenesis and a predisposition for apoptosis, wherein said cell
line is made by culturing a cell obtained from the nonhuman mammal of claim 56.

10   49. A method of determining whether a composition affects expression
of a gene selected from the group consisting of the *p53* gene and a gene encoding a
MutS homolog, said method comprising

  administering said composition to a first non-human mammal which is
nullizygous for one of said *p53* gene and said gene encoding a MutS homolog;
15   comparing a phenotype of said non-human mammal with said phenotype of a
second non-human mammal of the same type which is not nullizygous for said one of
said *p53* gene and said gene encoding a MutS homolog, wherein said phenotype is
selected from the group consisting of inappropriate fetal apoptosis and a predisposition
for carcinogenesis;

20   whereby a difference between said phenotype of said first non-human mammal
and said phenotype of said second non-human mammal is an indication that said
composition affects expression of the other of said *p53* gene and said gene encoding a
MutS homolog.

   50. A method of determining whether a composition affects expression
25 of a gene selected from the group consisting of the *p53* gene and a gene encoding a
MutS homolog, said method comprising

administering said composition to a first cell derived from a non-human

mammal which is nullizygous for one of said *p53* gene and said gene encoding a MutS

homolog;

comparing a phenotype of said first cell with said phenotype of a second cell

5   derived from a non-human mammal of the same type which is not nullizygous for said

one of said *p53* gene and said gene encoding a MutS homolog, wherein said phenotype

is selected from the group consisting of inappropriate fetal apoptosis and a

predisposition for carcinogenesis;

whereby a difference between said phenotype of said first cell and said

10   phenotype of said second cell is an indication that said composition affects expression

of the other of said *p53* gene and said gene encoding a MutS homolog.

51.   A composition comprising a human MutS homolog fragment,

wherein said fragment comprises a MutS homolog interaction region.

52.   A method of inhibiting association of a first human MutS homolog

15   and a second human MutS homolog, said method comprising contacting at least one of

said first human MutS homolog and said second human MutS homolog with a human

MutS homolog fragment comprising a MutS homolog interaction region, whereby

association of said first human MutS homolog and said second human MutS homolog

is inhibited.

20   53.   A composition comprising substantially purified hMSH5.

54.   A composition comprising an isolated nucleic acid encoding

hMSH5.

55.   A method of modifying a mismatched duplex DNA, said method

25   comprising contacting an MSH dimer and said mismatched duplex DNA in the

presence of a binding solution comprising ADP, wherein the concentration of ADP in

said binding solution is at least about ten times the concentration of ATP, if ATP is

present in said binding solution, whereby said MSH dimer associates with the

mismatched region of said mismatched duplex DNA, thereby modifying said

5   mismatched duplex DNA.

Fig. IA

Fig. IB

Fig. IC

Fig. ID

Fig. IE

Fig. IF

Fig.
2A

Fig.
2B

Fig. 2C

Fig.2D

Figure
3

Fig. 4A

Fig. 4B

Fig. 5A

Fig. 5B

Fig. 6A
Fig. 6B
Fig. 6C
Fig. 6D

Fig. 7
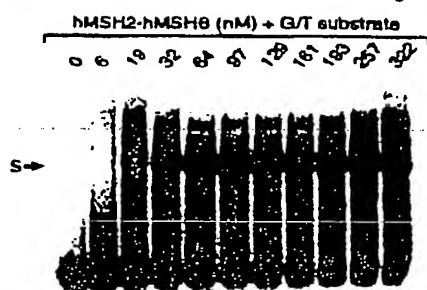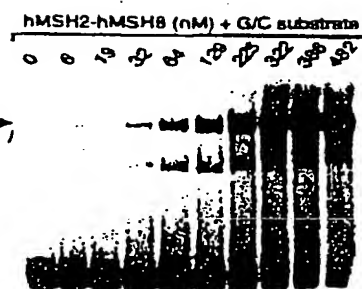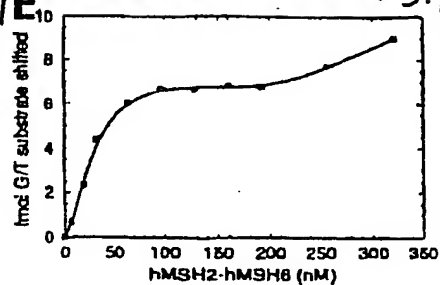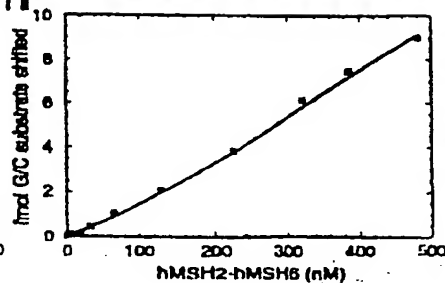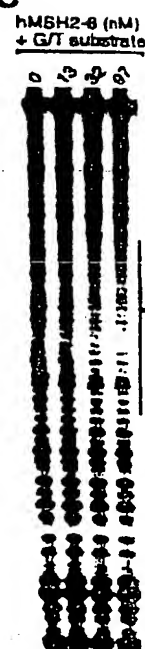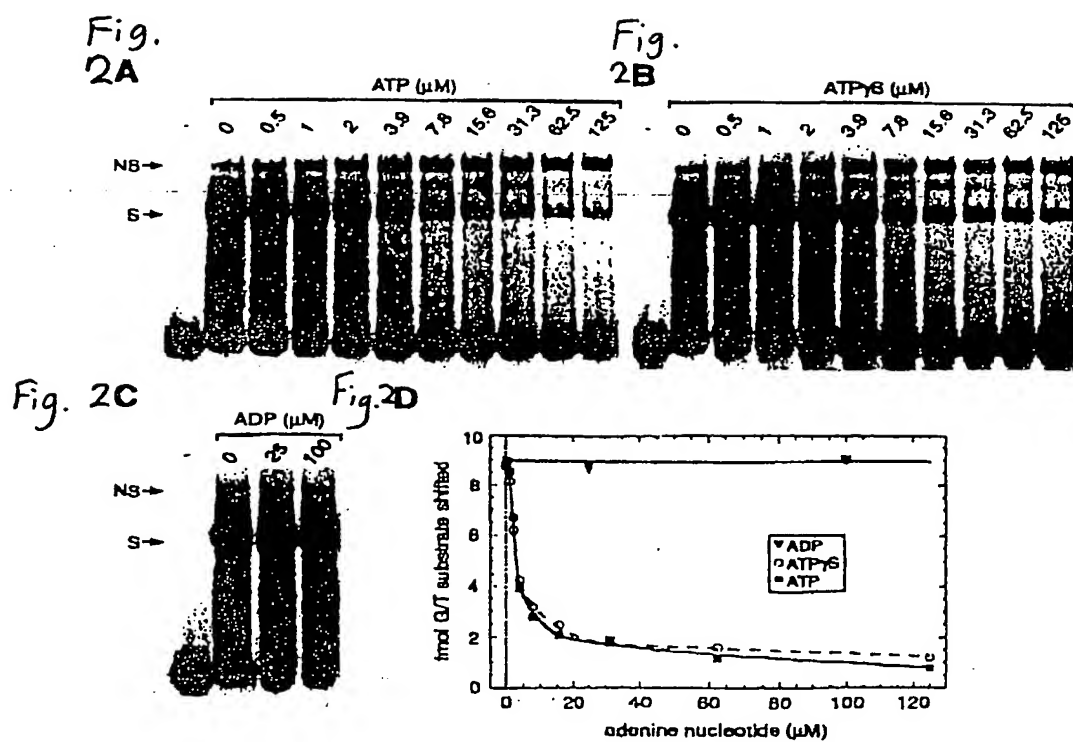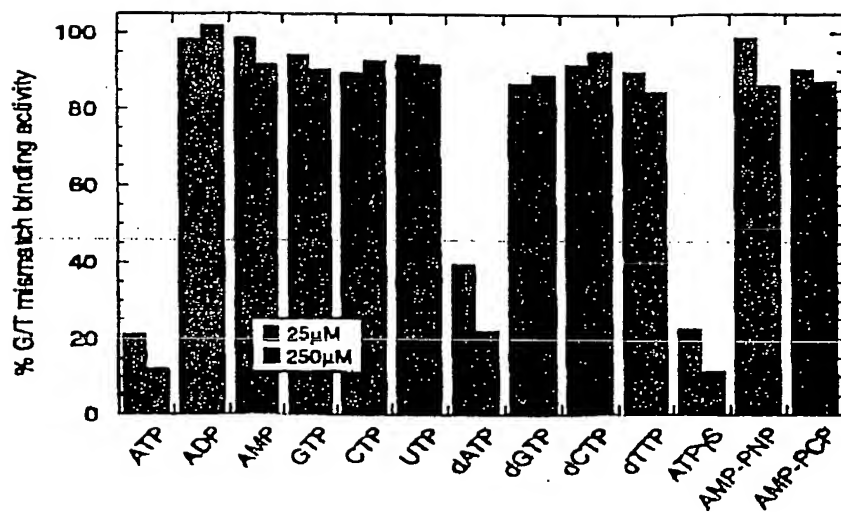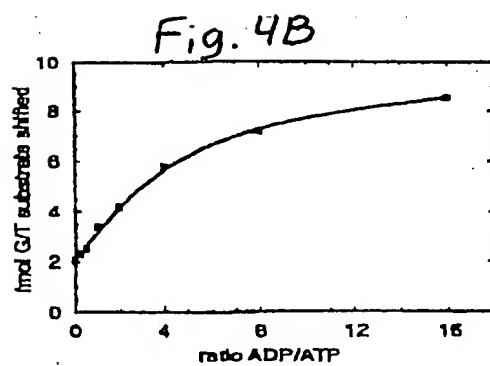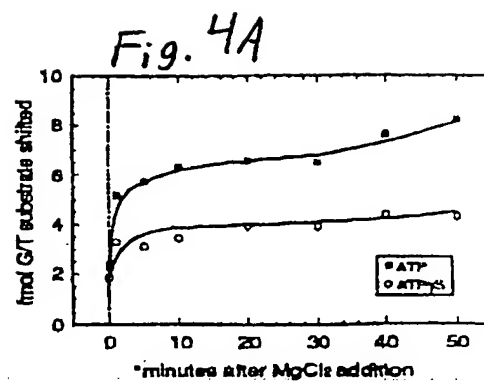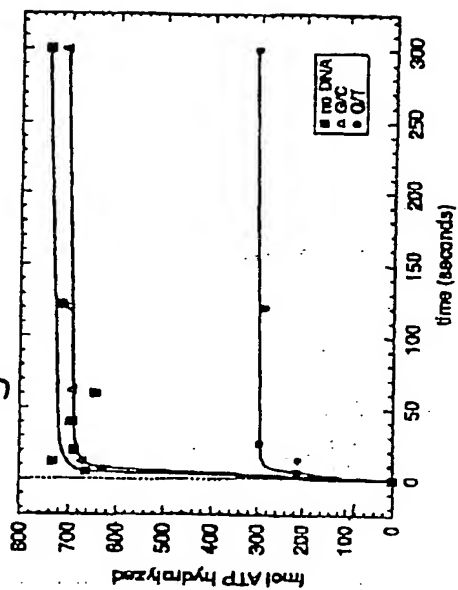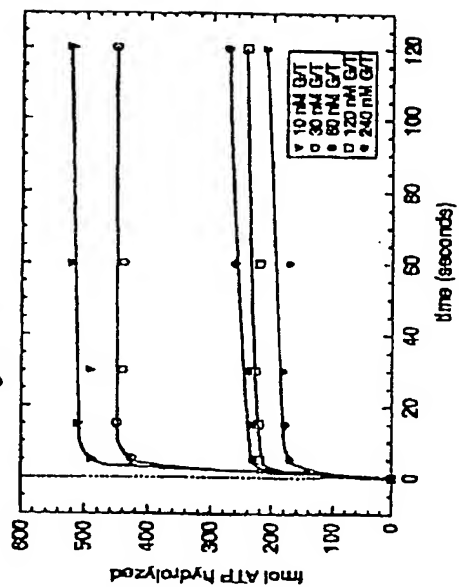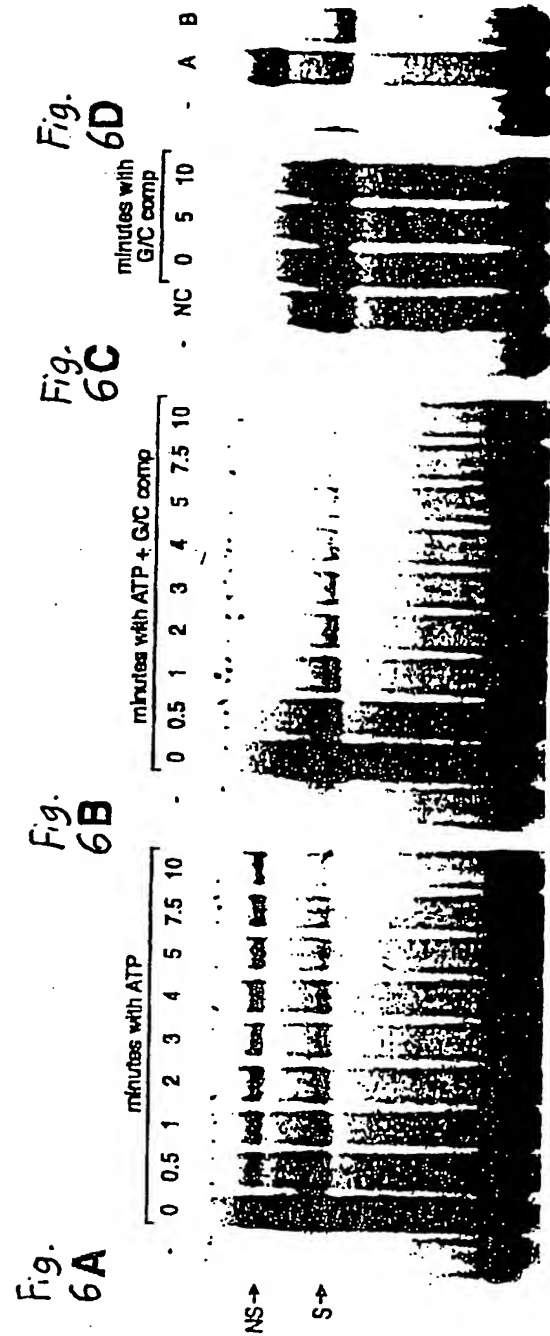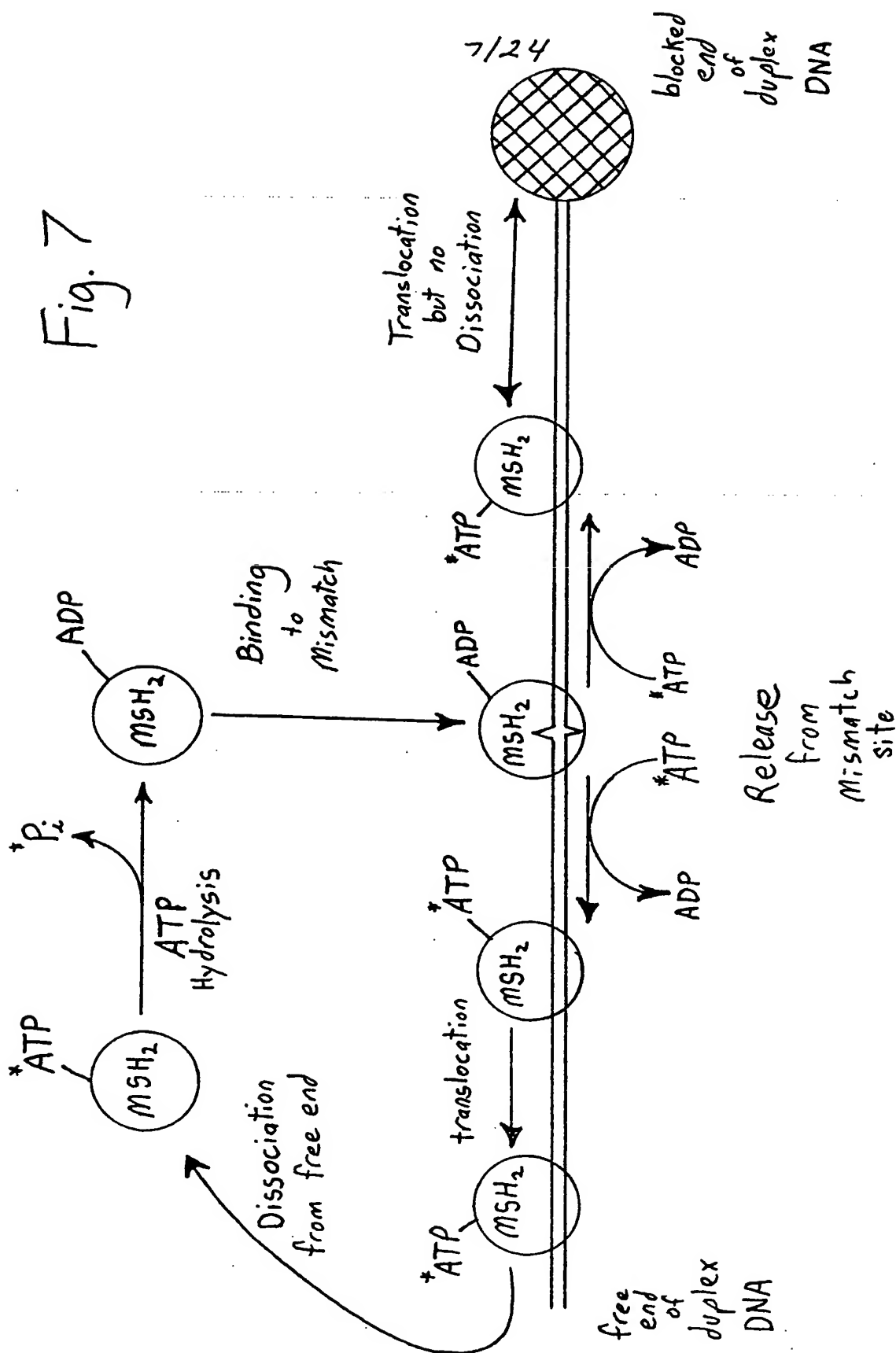
Figure 8

```
SEQ ID NO: 2
CCTGGTACCT CGAGCGATCA AGCTTGGTGG AATTCGCCG

SEQ ID NO: 3
CCTGGTACCT CGAGCGATCG AGCTTGGTGG AATTCGCCG

SEQ ID NO: 5
ACTATAGGGC GAATTGGGTA CCGCTGAATT GCACCGAGCT CGATCCTCGA TGATCCTAAG
CTAAGCTTCA GCTCCAGCTT T

SEQ ID NO: 6
ACTATAGGGC GAATTGGGTA CCGCTGAATT GCACCGAGCT TGATCCTCGA TGATCCTAAG
CTAAGCTTCA GCTCCAGCTT T
```

Figure
9A

Figure
9B

Figure
9C

Figure

9D
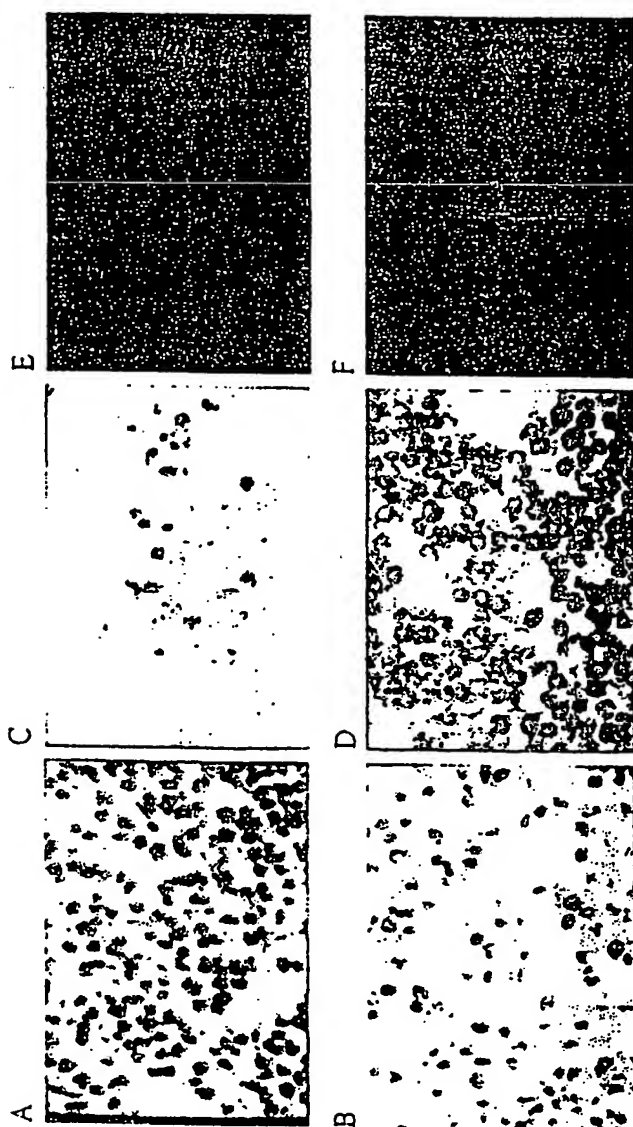
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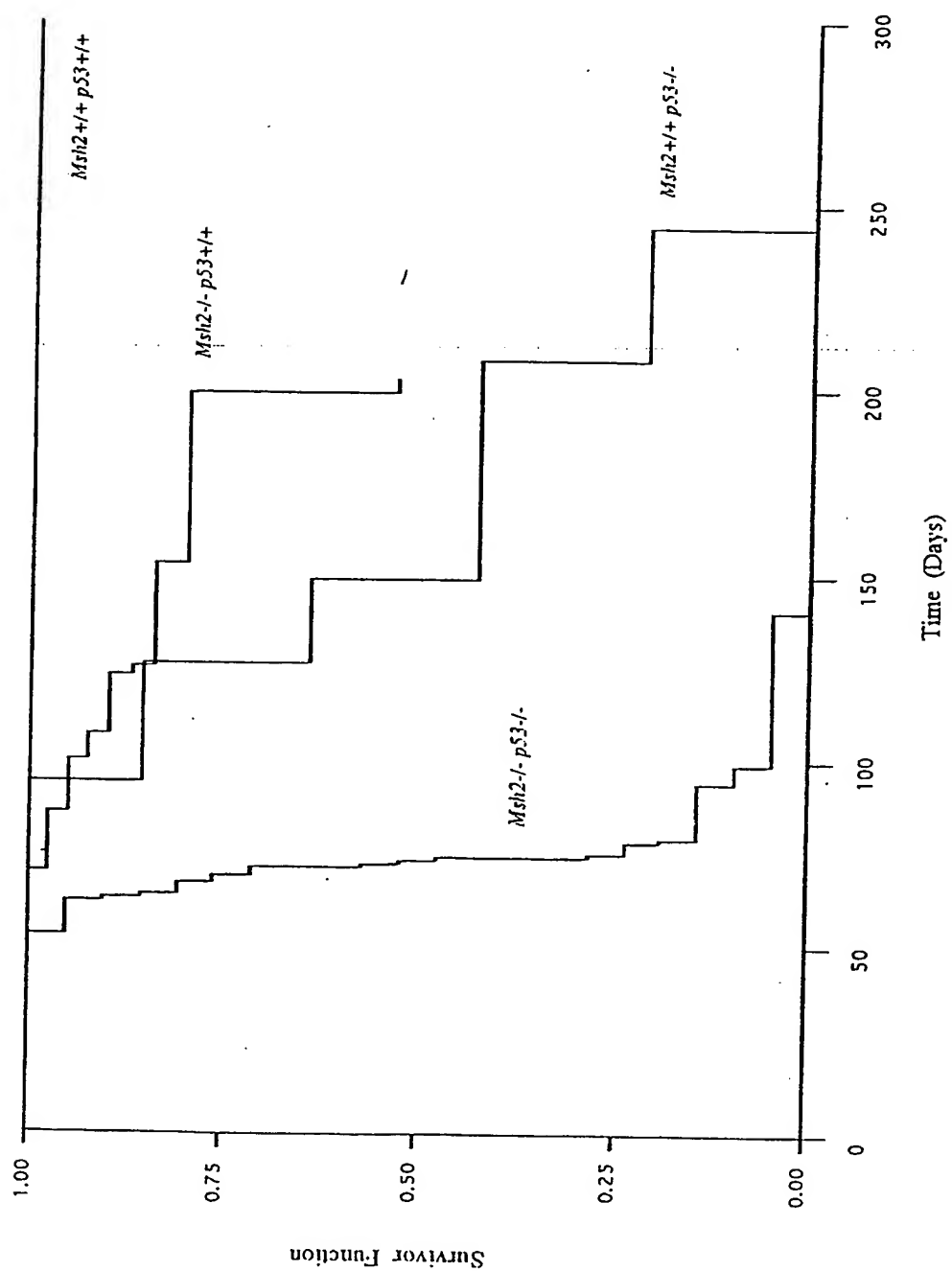
Figure 11

Fig. 12
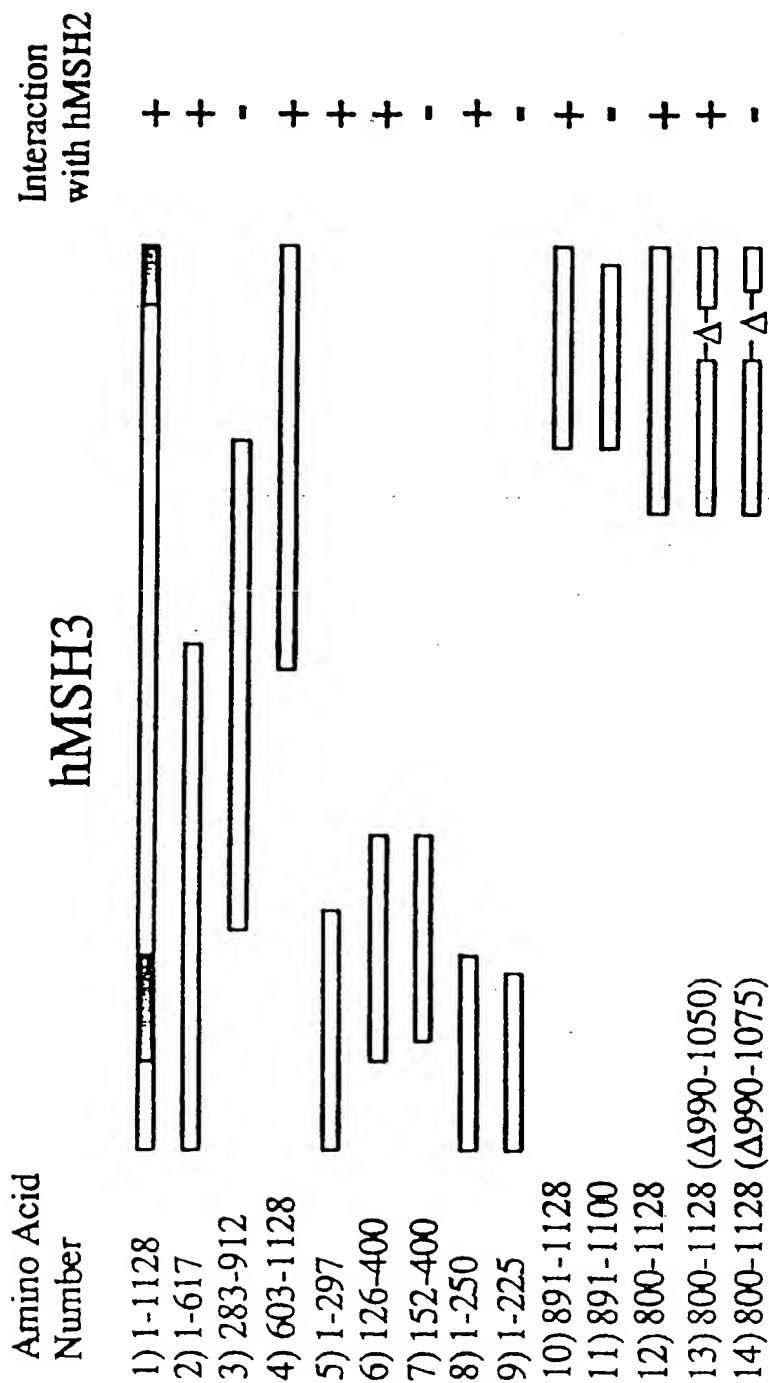
Fig. 13

Fig. 14

Fig. 15

Fig. 16

Fig. 17

Fig. 18

21/24

Figure 19A

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1                                                  CAGAAACCTCATACTTCTCGGGTCAGGGAAGGTT
35        TGGGAGGGCGTGGCGGTCGGTCAGCGGGGCGTTCTCCCACCTGTAGCGACTCAGAGCCTCCAAGCTC


          Met Ala Ser Leu Gly Ala Asn Pro Arg Arg Thr Pro Gln Gly Pro Arg Pro   17
102       ATG GCC TCC TTA GGA GCG AAC CCA AGG AGG ACA CCG CAG GGA CCG AGA CCT


          Gly Ala Ala Ser Ser Gly Phe Pro Ser Pro Ala Pro Val Pro Gly Pro Arg   34
154       GGG GCG GCC TCC TCC GGC TTC CCC AGC CCG GCC CCA GTG CCG GGC CCC AGG


          Glu Ala Glu Glu Glu Glu Val Glu Glu Glu Glu Glu Leu Ala Glu Ile His   51
205       GAG GCC GAG GAG GAG GAA GTC GAG GAG GAG GAG GAG CTG GCC GAG ATC CAT


          Leu Cys Val Leu Trp Asn Ser Gly Tyr Leu Gly Ile Ala Tyr Tyr Asp Thr   68
256       CTG TGT GTG CTG TGG AAT TCA GGA TAC TTG GGC ATT GCC TAC TAT GAT ACT


          Ser Asp Ser Thr Ile His Phe Met Pro Asp Ala Pro Asp His Glu Ser Leu   85
307       AGT GAC TCC ACT ATC CAC TTC ATG CCA GAT GCC CCA GAC CAC GAG AGC CTC


          Lys Leu Leu Gln Arg Val Leu Asp Glu Ile Asn Pro Gln Ser Val Val Thr   102
358       AAG CTT CTC CAG AGA GTT CTG GAT GAG ATC AAT CCC CAG TCT GTT GTT ACG


          Ser Ala Lys Gln Asp Glu Asn Met Thr Arg Phe Leu Gly Lys Leu Ala Ser   119
409       AGT GCC AAA CAG GAT GAG AAT ATG ACT CGA TTT CTG GGA AAG CTT GCC TCC


          Gln Glu His Arg Glu Pro Lys Arg Pro Glu Ile Ile Phe Leu Pro Ser Val   136
460       CAG GAG CAC AGA GAG CCT AAA AGA CCT GAA ATC ATA TTT TTG CCA AGT GTG


          Asp Phe Gly Leu Glu Ile Ser Lys Gln Arg Leu Leu Ser Gly Asn Tyr Ser   153
511       GAT TTT GGT CTG GAG ATA AGC AAA CAA CGC CTC CTT TCT GGA AAC TAC TCC


          Phe Ile Pro Asp Ala Met Thr Ala Thr Glu Lys Ile Leu Phe Leu Ser Ser   170
562       TTC ATC CCA GAC GCC ATG ACT GCC ACT GAG AAA ATC CTC TTC CTC TCT TCC


          Ile Ile Pro Phe Asp Cys Leu Leu Thr Val Arg Ala Leu Gly Gly Leu Leu   187
613       ATT ATT CCC TTT GAC TGC CTC CTC ACA GTT CGA GCA CTT GGA GGG CTG CTG


          Lys Phe Leu Gly Arg Arg Arg Ile Gly Val Glu Leu Glu Asp Tyr Asn Val   204
664       AAG TTC CTG GGT CGA AGA AGA ATC GGG GTT GAA CTG GAA GAC TAT AAT GTC


          Ser Val Pro Ile Leu Gly Phe Lys Lys Phe Met Leu Thr His Leu Val Asn   221
715       AGC GTC CCC ATC CTG GGC TTT AAG AAA TTT ATG TTG ACT CAT CTG GTG AAC


          Ile Asp Gln Asp Thr Tyr Ser Val Leu Gln Ile Phe Lys Ser Glu Ser His   238
766       ATA GAT CAA GAC ACT TAC AGT GTT CTA CAG ATT TTT AAG AGT GAG TCT CAC


          Pro Ser Val Tyr Lys Val Ala Ser Gly Leu Lys Glu Gly Leu Ser Leu Phe   255
817       CCC TCA GTG TAC AAA GTG GCC AGT GGA CTG AAG GAG GGG CTC AGC CTC TTT


          Gly Ile Leu Asn Arg Cys His Cys Lys Trp Gly Glu Lys Leu Leu Arg Leu   272
868       GGA ATC CTC AAC AGA TGC CAC TGT AAG TGG GGA GAG AAG CTG CTC AGG CTA
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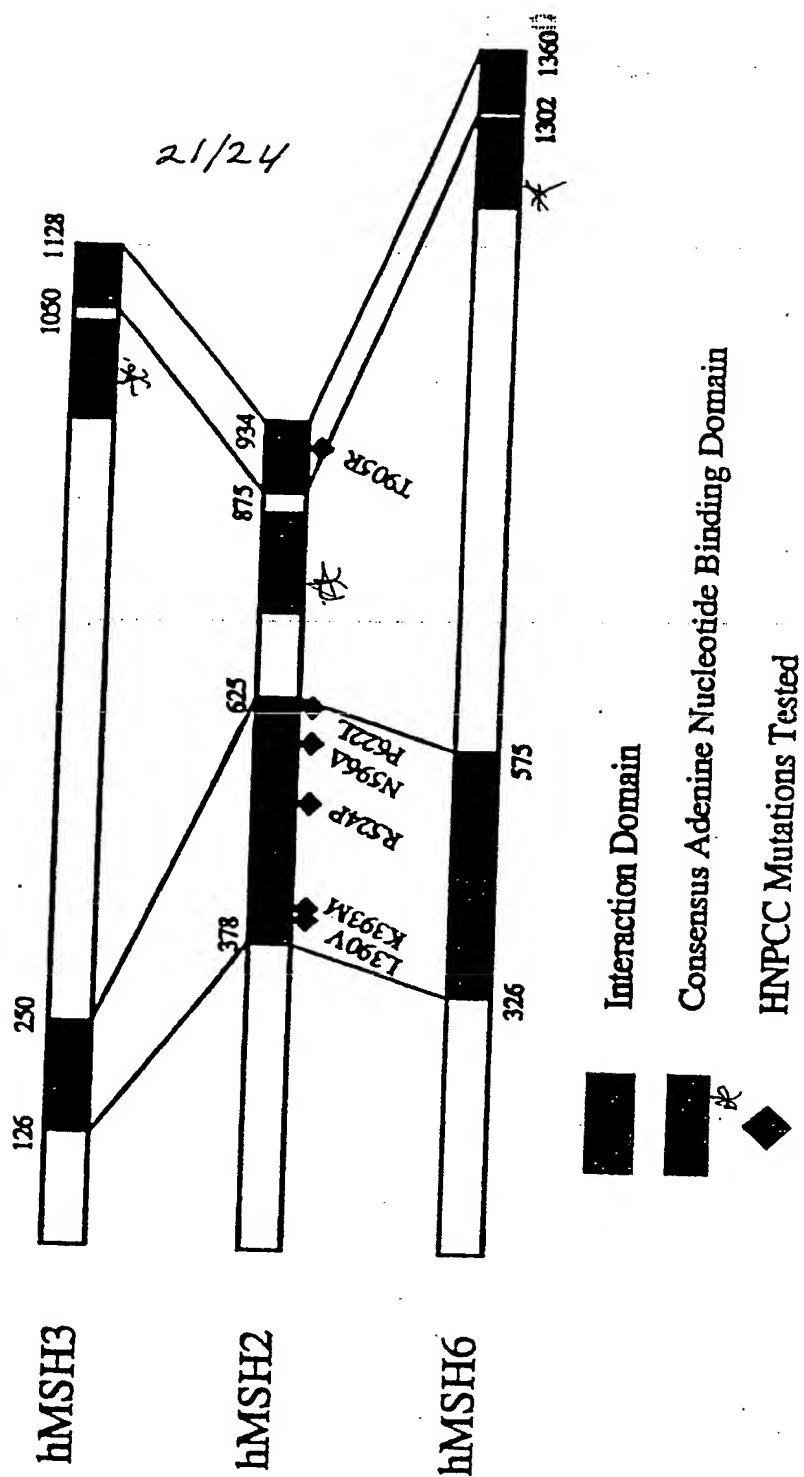
Figure 19B

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      Trp Phe Thr Arg Pro Thr His Asp Leu Gly Glu Leu Ser Ser Arg Leu Asp  289
 919  TGG TTC ACA CGT CCG ACT CAT GAC CTG GGG GAG CTC AGT TCT CGT CTG GAC

      Val Ile Gln Phe Phe Leu Leu Pro Gln Asn Leu Asp Met Ala Gln Met Leu  306
 970  GTC ATT CAG TTT TTT CTG CTG CCC CAG AAT CTG GAC ATG GCT CAG ATG CTG

      His Arg Leu Leu Gly His Ile Lys Asn Val Pro Leu Ile Leu Lys Arg Met  323
1021  CAT CGG CTC CTG GGT CAC ATC AAG AAC GTG CCT CTG ATT CTG AAA CGC ATG

      Lys Leu Ser His Thr Lys Val Ser Asp Trp Gln Val Leu Tyr Lys Thr Val  340
1072  AAG TTG TCC CAC ACC AAG GTC AGC GAC TGG CAG GTT CTC TAC AAG ACT GTG

      Tyr Ser Ala Leu Gly Leu Arg Asp Ala Cys Arg Ser Leu Pro Gln Ser Ile  357
1123  TAC AGT GCC CTG GGC CTG AGG GAT GCC TGC CGC TCC CTG CCG CAG TCC ATC

      Gln Leu Phe Arg Asp Ile Ala Gln Glu Phe Ser Asp Asp Leu His His Ile  374
1174  CAG CTC TTT CGG GAC ATT GCC CAA GAG TTC TCT GAT GAC CTG CAC CAT ATC

      Ala Ser Leu Ile Gly Lys Val Val Asp Phe Glu Gly Ser Leu Ala Glu Asn  391
1225  GCC AGC CTC ATT GGG AAA GTA GTG GAC TTT GAG GGC AGC CTT GCT GAA AAT

      Arg Phe Thr Val Leu Pro Asn Ile Asp Pro Glu Ile Asp Glu Lys Lys Arg  408
1276  CGC TTC ACA GTC CTC CCC AAC ATA GAT CCT GAA ATT GAT GAG AAA AAG CGA

      Arg Leu Met Gly Leu Pro Ser Phe Leu Thr Glu Val Ala Arg Lys Glu Leu  425
1327  AGA CTG ATG GGA CTT CCC AGT TTC CTT ACT GAG GTT GCC CGC AAG GAG CTG

      Glu Asn Leu Asp Ser Arg Ile Pro Ser Cys Ser Val Ile Tyr Ile Pro Leu  442
1378  GAG AAT CTG GAC TCC CGT ATT CCT TCA TGC AGT GTC ATC TAC ATC CCT CTG

      Ile Gly Phe Leu Leu Ser Ile Pro Arg Leu Pro Ser Met Val Glu Ala Ser  459
1429  ATT GGC TTC CTT CTT TCT ATT CCC CGC CTG CCT TCC ATG GTA GAG GCC AGT

      Asp Phe Glu Ile Asn Gly Leu Asp Phe Met Phe Leu Ser Glu Glu Lys Leu  476
1480  GAC TTT GAG ATT AAT GGA CTG GAC TTC ATG TTT CTC TCA GAG GAG AAG CTG

      His Tyr Arg Ser Ala Arg Thr Lys Glu Leu Asp Ala Leu Leu Gly Asp Leu  493
1531  CAC TAT CGT AGT GCC CGA ACC AAG GAG CTG GAT GCA TTG CTG GGG GAC CTG

      His Cys Glu Ile Arg Asp Gln Glu Thr Leu Leu Met Tyr Gln Leu Gln Cys  510
1582  CAC TGC GAG ATC CGG GAC CAG GAG ACG CTG CTG ATG TAC CAG CTA CAG TGC

      Gln Val Leu Ala Arg Ala Ala Val Leu Thr Arg Val Leu Asp Leu Ala Ser  527
1633  CAG GTG CTG GCA CGA GCA GCT GTC TTA ACC CGA GTA TTG GAC CTT GCC TCC

      Arg Leu Asp Val Leu Leu Ala Leu Ala Ser Ala Ala Arg Asp Tyr Gly Tyr  544
1684  CGC CTG GAC GTC CTG CTG GCT CTT GCC AGT GCT GCC CGG GAC TAT GGC TAC

      Ser Arg Pro Arg Tyr Ser Pro Gln Val Leu Gly Val Arg Ile Gln Asn Gly  561
1735  TCA AGG CCG CGT TAC TCC CCA CAA GTC CTT GGG GTA CGA ATC CAG AAT GGC
```

Figure 19C

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     Arg His Pro Leu Met Glu Leu Cys Ala Arg Thr Phe Val Pro Asn Ser Thr   578
1786 AGA CAT CCT CTG ATG GAA CTC TGT GCC CGA ACC TTT GTG CCC AAC TCC ACA

     Glu Cys Gly Gly Asp Lys Gly Arg Val Lys Val Ile Thr Gly Pro Asn Ser   595
1837 GAA TGT GGT GGG GAC AAA GGG AGG GTC AAA GTC ATC ACT GGA CCC AAC TCA

     Ser Gly Lys Ser Ile Tyr Leu Lys Gln Val Gly Leu Ile Thr Phe Met Ala   612
1888 TCA GGG AAG AGC ATA TAC CTC AAA CAG GTA GGC TTG ATC ACA TTC ATG GCC

     Leu Val Gly Ser Phe Val Pro Ala Glu Glu Ala Glu Ile Gly Ala Val Asp   629
1939 CTG GTA GGC AGC TTT GTG CCA GCA GAG GAG GCC GAA ATT GGG GCA GTA GAC

     Ala Ile Phe Thr Arg Ile His Ser Cys Glu Ser Ile Ser Leu Gly Leu Ser   646
1990 GCC ATC TTC ACA CGA ATT CAT AGC TGC GAA TCC ATC TCC CTT GGC CTC TCC

     Thr Phe Met Ile Asp Leu Asn Gln Val Ala Lys Ala Val Asn Asn Ala Thr   663
2041 ACC TTC ATG ATC GAC CTC AAC CAG GTG GCG AAA GCA GTG AAC AAT GCC ACT

     Ala Gln Ser Leu Val Leu Ile Asp Glu Phe Gly Lys Gly Thr Asn Thr Val   680
2092 GCA CAG TCG CTG GTC CTT ATT GAT GAA TTT GGA AAG GGA ACC AAC ACG GTG

     Asp Gly Leu Ala Leu Leu Ala Ala Val Leu Arg His Trp Leu Ala Arg Gly   697
2143 GAT GGG CTC GCG CTT CTG GCC GCT GTG CTC CGA CAC TGG CTG GCA CGT GGA

     Pro Thr Cys Pro His Ile Phe Val Ala Thr Asn Phe Leu Ser Leu Val Gln   714
2194 CCC ACA TGC CCC CAC ATC TTT GTG GCC ACC AAC TTT CTG AGC CTT GTT CAG

     Leu Gln Leu Leu Pro Gln Gly Pro Leu Val Gln Tyr Leu Thr Met Glu Thr  731
2245 CTA CAA CTG CTG CCA CAA GGG CCC CTG GTG CAG TAT TTG ACC ATG GAG ACC

     Cys Glu Asp Gly Asn Asp Leu Val Phe Phe Tyr Gln Val Cys Glu Gly Val   748
2296 TGT GAG GAT GGC AAC GAT CTT GTC TTC TTC TAT CAG GTT TGC GAA GGT GTT

     Ala Lys Ala Ser His Ala Ser His Thr Ala Ala Gln Ala Gly Leu Pro Asp   765
2347 GCG AAG GCC AGC CAT GCC TCC CAC ACA GCT GCC CAG GCT GGG CTT CCT GAC

     Lys Leu Val Ala Arg Gly Lys Glu Val Ser Asp Leu Ile Arg Ser Gly Lys   782
2398 AAG CTT GTG GCT CGT GGC AAG GAG GTC TCA GAC TTG ATC CGC AGT GGA AAA

     Pro Ile Lys Pro Val Lys Asp Leu Leu Lys Lys Asn Gln Met Glu Asn Cys   799
2449 CCC ATC AAG CCT GTC AAG GAT TTG CTA AAG AAG AAC CAA ATG GAA AAT TGC

     Gln Thr Leu Val Asp Lys Phe Met Lys Leu Asp Leu Glu Asp Pro Asn Leu   816
2500 CAG ACA TTA GTG GAT AAG TTT ATG AAA CTG GAT TTG GAA GAT CCT AAC CTG

     Asp Leu Asn Val Phe Met Ser Gln Glu Val Leu Pro Ala Ala Thr Ser Ile   833
2551 GAC TTG AAC GTT TTC ATG AGC CAG GAA GTG CTG CCT GCT GCC ACC AGC ATC

     Leu Stop         834
2602 CTC TGA GAGTCCTTCCAGTGTCCTCCCCAGCCTCCTGAGACTCCGGTGGGCTGCCATGCCCTCTT
2667 TGTTTCCTTATCTCCCTCAGACGCAGAGTTTTTAGTTTCTCACAATTCTAATGTAATAATATATCTT
2734 AA
```

INTERNATIONAL SEARCH REPORT

A. CLASSIFICATION OF SUBJECT MATTER

IPC(6) :C07H 21/04; C07K 14/435; C12Q 1/68; C12P 19/34

US CL :435/6, 91.1, 91.2, 91.5; 530/350; 536/23.1

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

U.S. : 435/6, 91.1, 91.2, 91.5; 530/350; 536/23.1

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

APS, DIALOG ONESEARCH: MutS, mismatch repair

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	ACHARYA et al. hMSH2 Forms Specific Mispair-Binding Complexes With hMSH3 and hMSH6, Proc. Natl. Acad. Sci. USA. November 1996, Vol. 93, pages 13629-13634, esp. pages 13631-13633.	1-34, 36, 38, 40, 41 55
Y	FISHEL et al. Binding of Mismatched Microsatellite DNA Sequences by the Human MSH2 Protein. Science. 25 November 1994, Vol. 266, pages 1403-1405, see entire document.	1-34, 36, 38, 40, 41, 55
Y	FISHEL et al. Purified Human MSH2 Protein Binds to DNA Containing Mismatched Nucleotides, Cancer Res. November 1994, Vol. 84, pages 5539-5542, see entire document	1-34, 36, 38, 40, 41, 55

☒ Further documents are listed in the continuation of Box C. ☐ See patent family annex.

*	Special categories of cited documents:	"T"	later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
"A"	document defining the general state of the art which is not considered to be of particular relevance		
"E"	earlier document published on or after the international filing date	"X"	document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone
"L"	document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)	"Y"	document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art
"O"	document referring to an oral disclosure, use, exhibition or other means		
"P"	document published prior to the international filing date but later than the priority date claimed	"&"	document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
18 NOVEMBER 1998	23 DEC 1998

Name and mailing address of the ISA/US	Authorized officer
Commissioner of Patents and Trademarks Box PCT Washington, D.C. 20231	STEPHANIE ZITOMER, PHD
Facsimile No. (703) 305-3230	Telephone No. (703) 308-0196

Form PCT/ISA/210 (second sheet)(July 1992)*

C (Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	CHI et al. Purification and Characterization of MSH1, a Yeast Mitochondrial Protein That Binds to DNA Mismatches. J. Biol. Chem. 25 November 1994, Vol.269, No. 47, pages 29984-29992, esp. pages 29987-29990.	1-34, 36, 38, 40, 41, 55
Y	CHI et al. The effect of DNA Mismatches on the ATPase Activity of MSH1, a Protein in Yeast Mitochondria That Recognizes DNA Mismatches, J. Biol. Chem. 25 November 1994, Vol. 269, No. 47 pages 29993-29997, esp. pages 29994-29996.	1-34, 36, 38, 40, 41, 55
Y	FEARON et al. A Genetic Model for Colorectal Tumorigenesis. Cell. 01 June 1990, Vol. 61, pages 759-767, esp. pages 759-780.	15-19, 38
Y	FISHEL et al. The Human Mutator Gene Homolog MSH2 and Its Association With Hereditary Nonpolyposis Colon Cancer. Cell. 03 December 1993, Vol. 75, pages 1027-1038, see entire document.	15-19, 38
Y	DRUMMOND et al. Isolation of an hMSH2-p160 Heterodimer That Restores DNA Mismatch Repair to Tumor Cells. Science. 30 June 1995, Vol. 268, pages 1909-1912, see entire document.	15-19, 38

Form PCT/ISA/210 (continuation of second sheet)(July 1992)★

INTERNATIONAL SEARCH REPORT

International application No.

PCT/US98/17914

Box I Observations where certain claims were found unsearchable (Continuation of item 1 of first sheet)

This international report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. ☐ Claims Nos.:
   because they relate to subject matter not required to be searched by this Authority, namely:

2. ☐ Claims Nos.:
   because they relate to parts of the international application that do not comply with the prescribed requirements to such an extent that no meaningful international search can be carried out, specifically:

3. ☐ Claims Nos.:
   because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

Box II Observations where unity of invention is lacking (Continuation of item 2 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:

   Please See Extra Sheet.

1. ☐ As all required additional search fees were timely paid by the applicant, this international search report covers all searchable claims.

2. ☐ As all searchable claims could be searched without effort justifying an additional fee, this Authority did not invite payment of any additional fee.

3. ☐ As only some of the required additional search fees were timely paid by the applicant, this international search report covers only those claims for which fees were paid, specifically claims Nos.:

4. ☒ No required additional search fees were timely paid by the applicant. Consequently, this international search report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:
   1-34, 36, 38, 40, 41, 43 and 55

Remark on Protest   ☐ The additional search fees were accompanied by the applicant's protest.

   ☐ No protest accompanied the payment of additional search fees.

Form PCT/ISA/210 (continuation of first sheet(1))(July 1992)★

BOX II. OBSERVATIONS WHERE UNITY OF INVENTION WAS LACKING
This ISA found multiple inventions as follows:

This application contains the following inventions or groups of inventions which are not so linked as to form a single inventive concept under PCT Rule 13.1. In order for all inventions to be searched, the appropriate additional search fees must be paid.

Group I, claim(s)1-34, 36, 38, 40, 41, 43 and 55,
drawn to a method and kit for modifying or detecting a mismatched duplex DNA;
Group II, claim(s) 35, 37, 39 and 42, drawn to a method and composition for segregating or fractionating duplex DNAs;
Group III, claim(s) 44-50, drawn to a nonhuman mammal and methods of making and using same;
Group IV, claim(s) 51-53, drawn to a protein composition and method of treatment;
Group V, claim(s) 54, drawn to a nucleic acid composition.

The inventions listed as Groups I-V do not relate to a single inventive concept under PCT Rule 13.1 because, under PCT Rule 13.2, they lack the same or corresponding special technical features for the following reasons: The special technical feature of the claimed invention is the MutS homolog property of binding to mismatched duplex DNA. As this property and the role of ADP/ATP in the binding were known in the prior art as documented by the Fishel et al. review in Current Opinion in Genetics and Development published in early 1997 and the references cited therein, the claims are considered not to avoid the prior art and, therefore, to lack unity of invention.

The methods of invention Groups I-IV differ from one another in having different method steps and different functions/results and using different elements. In the Group I method an MSH dimer binds and thereby modifies a mismatched duplex DNA. Mismatched duplex DNA bound by an MSH dimer is segregated or mixed DNA populations are fractionated in the method of Group II. The Group III methods involve construction of a nonhuman mammal and use of the mammal for screening compounds and do not involve use of MSH. The method of Group IV is a treatment method which uses only a fragment of MSH. The last Group, V, is a nucleic acid which is not involved in any of the Group I-IV methods.

Form PCT/ISA/210 (extra sheet)(July 1992)★